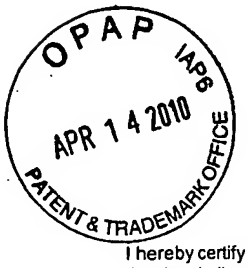

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BOARD OF PATENT APPEALS AND INTERFERENCES

CERTIFICATE OF MAILING

I hereby certify that this Appeal Brief and the documents referred to as enclosed therein are being deposited with the United States Postal Service on the date indicated below with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Bethany Crandell — Bethany Crandell

7 April, 2010 — Date of Deposit

Applicant: Markou et al.	Art Unit: 1627
Serial No.: 10/527,525	Examiner: Kendra Carter
I.A. Filing Date: Sep. 10, 2003	Confirmation No.: 3218
Title: METHODS FOR TREATING DISORDERS ASSOCIATED WITH mGLU RECEPTORS INCLUDING ADDICTION AND DEPRESSION	Our Ref.: TSRI 897.1

APPEAL BRIEF

MAIL STOP: Appeal Brief-Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Applicants appeal the Final Office Action, dated December 15, 2009 ("Office Action," attached as Ref. 17), and the rejection of claims 1-3, 6, 7, 9, 16, 27, 28 and 32 in the above-referenced patent application.

Table of Contents

Real Party in Interest	Page 3
Related Appeals and Interferences	Page 4
Status of Claims	Page 5
Status of Amendments	Page 6
Summary of Claimed Subject Matter	Pages 7
Grounds of Rejection to be Reviewed on Appeal	Page 8
Arguments	Pages 9-21
Summary of Arguments	Page 22
Claims Appendix	Pages 23-27
Evidence Appendix	Page 28 and Documents 1-17 cited therein
Related Proceedings Appendix	Page 29

I. Real Party in Interest

The present application has been assigned by the inventors to The Scripps Research Institute, which is the real party in interest.

II. Related Appeals and Interferences

There are no related appeals or interferences.

III. Status of Claims

Claims 1-3, 6-7, 9-10, 14-17, 19, 27-28 and 32 are pending.

Claims 4, 5, 8, 11-13, 18, 20-26, 29-31, and 33 were canceled by Applicants

Claims 10, 14, 15, 17 and 19 are withdrawn from consideration by the Examiner

Claims 1-3, 6, 7, 9, 16, 27, 28 and 32 are rejected.

Claims 1-3, 6, 7, 9, 27, 28 and 32 are on appeal.

IV. Status of Amendments

All amendments previously submitted by Appellants have been entered.

No amendment was filed by Appellants subsequent to the noted Office Action.

V. Summary of Claimed Subject Matter

The claimed inventions relate to treatment of drug dependence/addictions and withdrawal symptoms associated therewith. Specifically, Independent claim 1 is directed to a method of treating drug dependence, such as nicotine addiction, alcohol addiction, opiate addiction and cocaine addiction. The method entails the administration to a subject in need of treatment (1) a first antagonist for metabotropic glutamate receptor 2 (mGluR 2) and/or metabotropic glutamate receptor 3 (mGluR 3) and (2) a second antagonist for metabotropic glutamate receptor 5 (mGluR 5). Claim 2 more specifically recites administration of a combination of a mGluR2 antagonist and a mGluR5 antagonist, while claim 3 specifies the combination of a mGluR3 antagonist and a mGluR5 antagonist. Claims 6, 7, and 9 depend from claim 1. Support for these claims is provided in the claims as originally filed and in the specification, e.g., at page 2, last paragraph to page 3, 4th paragraph; page 11, first two paragraphs; page 17, last paragraph to page 19, penultimate paragraph; and Example 3 at pages 64-79 (especially disclosures related to Experiment 3.5 at page 73, 1st paragraph; page 76, last paragraph; and page 79, first two paragraphs).

Claim 16 is directed to treating addictive disorders via the use of a combination that comprises a first active ingredient which is a mGluR2 antagonist or a mGluR3 antagonist and a second active ingredient which is a mGluR5 antagonist. Support for this claim is provided in the specification, e.g., at page 11, last paragraph to page 12, penultimate paragraph; and claim 16 as originally filed.

Similar to claims 1-3, independent claim 27 is also directed to treating addictive disorders including nicotine addiction and alcohol addiction. However, this claim specifies administration of a mGluR5 antagonist during a first time period and administration of a mGluR2 or mGluR3 antagonist during a second time period. Support for this claim is provided in the specification, e.g., page 23, last paragraph to page 14, second paragraph; and original claims 27-28 as filed.

VI. Grounds of rejection to be reviewed on appeal

Issue 1. Whether Claims 1-3, 6, 7 and 16 are unpatentable under 35 U.S.C. § 103(a) over Adam et al. (U.S. Patent No. 6,406,094; attached as Ref. 1) in view of Corsi et al. (U.S. Application 2003/0195139; attached as Ref. 2) or Chiamulera et al. (Nat. Neurosci. 4:873-874, 2001; attached as Ref. 3)?

Issue 2. Whether Claims 9, 27, 28 and 32 are unpatentable under 35 U.S.C. § 103(a) over Chiamulera et al. in view of Adam et al.?

VII. Argument

The Examiner rejected claims 1-3, 6, 7 and 16 under 35 U.S.C. § 103(a) as allegedly unpatentable over Adam et al. (U.S. Patent No. 6,406,094) in view of Corsi et al. (U.S. Application 2003/0195139) or Chiamulera et al. (Nat. Neurosci. 4:873-874, 2001). The Examiner additionally rejected claims 9, 27, 28 and 32 under 35 U.S.C. § 103(a) as allegedly unpatentable over Chiamulera et al. in view of Adam et al. and some other art cited in the subject specification. The rationale underlying the Examiner's the rejection of claims 1-3, 6, 7 and 16 can be summarized as follows.

(1) "Adam et al. teaches compounds that act as Group II (i.e., mGluR2 and 3) metabotropic glutamate receptor antagonist (see column 16, lines 47 and 48) and treat conditions such as nicotine addiction, and opiate addiction (see column 1, lines 54-56 and column 3, lines 20-24; . . ." (Office Action, paragraph bridging page 3 and page 4; emphasis added);

(2) Corsi et al. teaches treating substance dependence with an mGluR5 antagonist; and Chiamulera et al. teaches administration of a mGluR5 antagonist to decrease cocaine self-administration (Office Action, page 4, paragraphs 3 and 4);

(3) Because Adam et al. and Corsi et al. (or Chiamulera et al.) both teach methods for treating addictive disorders, the skilled artisan would be motivated to combine the methods taught in the cited art, i.e., using both an antagonist for mGluR2 or mGluR3 and an antagonist for mGluR5 in treating addictive disorders (Office Action, paragraph bridging page 4 and page 5).

The Examiner's rationale for rejecting claims 9, 27, 28 and 32 is based on assertions similar to that noted above, except for reference to some additional art for the

teaching of certain claim limitations present in these claims which are not disclosed in the primary references, i.e., Adam et al. and Chiamulera et al. Accordingly, unless otherwise noted, Appellants' arguments presented herein are intended to address both rejections (Issue 1 and Issue 2 set forth above).

As explained in detail below, Appellants respectfully traverse these rejections because no prima facie case of obviousness can be established on the evidence and reasoning provided by the Examiner. In brief, the prior art does not teach each and every element of the claimed inventions. In addition, the Examiner did not provide a sufficient reason or explicit analysis of why the skilled artisan would combine elements recited in the claimed inventions. Instead, teachings of the prior art, including references cited by the Examiner, would actually teach away from the claimed inventions. Furthermore, Appellants' inventions have demonstrated surprising results that would not have been expected from the prior art.

1. *The prior art did not teach every element of the claimed inventions*

It is well established that, to sustain an obviousness rejection, it is necessary to show "that all the claimed elements were known in the prior art and one skilled in the art could have combined the elements as claimed by known methods with no change in their respective functions, and the combination yielded nothing more than predictable results to one of ordinary skill in the art." See, e.g., *KSR International Co. v. Teleflex Inc.*, 550 U.S. ___, ___, 82 USPQ2d 1385, 1395 (2007). See also, MPEP §§ 706.02(j) and 2143. In the instant case, as elaborated in the following sections, the prior art did not teach all the elements recited in the claimed inventions. This is because, the prior art, including Adam et al. (one of the primary references relied on by the Examiner), did not teach treating addictive disorders with antagonist compounds for mGluR2 or mGluR3 as alleged by the Examiner. Rather, prior to the claimed inventions, it was not known in the art that antagonizing Group II glutamate receptors (i.e., mGluR2 or mGluR3) will be conducive to treating disorders mediated by metabotropic glutamate receptors. To the

contrary, the consensus review in the relevant scientific fields was that agonizing (not antagonizing) mGluR2 and mGlu3 is beneficial for treating drug dependence. On the other hand, Adam et al., which was the sole reference cited by the Examiner for the alleged teaching of treating addictive disorders with mGluR2/3 antagonists, merely alluded to possible therapeutic uses of certain antagonist compounds for mGluR2 and mGluR3. There was no actual data or other substantiation in Adam et al. to support the assertion.

A. **Prior art teaching of mGluR2/3 agonism for treating addictions**

The present inventors were the first to experimentally demonstrate that mGluR2/3 antagonists are effective in the treatment of drug dependence and withdrawal symptoms. The inventors' study was described in detail in Example 1 of the subject specification and also in a corresponding post-filing publication, Kenny et al., (J. Pharmacol. Exp. Ther. 306:1068-76, 2003; attached as Ref. 16). Prior to the claimed inventions, the consensus view in the scientific community on the connection between modulating mGluR2/3 receptors and reducing withdrawal symptoms was the opposite to that reflected by the results obtained by the present inventors. At that time, several research groups have shown that agonists (not antagonists) of mGluR2/3 were able to attenuate withdrawal symptoms and to treat morphine or nicotine dependence. The apparent difference between the prior art studies and the subject disclosure is explained in the specification, e.g., at page 43, middle paragraph; and page 45, 3rd paragraph to page 46, 1st paragraph.

Specifically, Helton et al. (Neuropharmacol. 36:1522-6, 1997; attached as Ref. 13) reported that the mGluR2/3 agonist LY354740, when administered to nicotine dependent rats, resulted in a dose-dependent attenuation of the enhanced auditory startle response following withdrawal from chronic nicotine exposure. Helton et al. further noted that their data indicate that the mGluR2/3 agonist could be effective in treating nicotine withdrawal symptoms during smoking cessation in humans. Similar

data suggesting the use of mGluR2/3 agonists in treating withdrawal symptoms were also reported in other scientific papers published prior to Appellants' inventions. For example, Vandergriff and Rasmussen (Neuropharmacol. 38:217-22, 1999; attached as Ref. 14) reported that the mGluR2/3 agonist LY354740 was able to reduce symptoms in morphine dependent rats following withdrawal from chronic morphine exposure, and suggested its potential therapeutic use in treating human opiate dependence. In another study, Fundytus and Coderre (Brit. J. Pharmacol. 121:511-4, 1997; attached as Ref. 15) reported that the mGLuR2/3 agonist DCG-IV was able to significantly attenuate withdrawal symptoms in morphine dependent rats. The authors suggested that activation of the mGluR receptors could reduce withdrawal symptoms in human patients.

Thus, it is readily apparent that, prior to the claimed inventions, scientific publications in the prior art taught that activation of mGluR2/3 receptors could produce beneficial effects in treating drug dependence (e.g., reducing withdrawal symptoms). By extension, one would understand that inhibition of mGluR2/3 receptors (e.g., via an antagonist compound) is likely to exacerbate withdrawal symptoms (or at best, to have no effect in ameliorating withdrawal symptoms). One would certainly not be motivated by the unsubstantiated speculation in Adam et al., as discussed below, to attempt treatment of drug dependence with an mGluR2/3 antagonist. To the contrary, the consensus view of the leading scientists in the relevant technical fields (as evidenced by the above-noted publications) would undoubtedly have led a skilled artisan away from such a treatment.

B. Disclosure of Adam et al.

The Examiner relied on Adam et al. for the alleged art teaching of the use of mGluR2/3 antagonists in treating addictive disorders. However, the Examiner's reliance on Adam et al. in rendering and maintaining the obviousness rejection of the claimed inventions in clearly unjustified. This is because Adam et al. did not actually

teach the use of mGluR2/3 antagonism for treating addictions. Instead, Adam et al. disclosed certain compounds which are purportedly Group II mGlu receptor antagonists. Adam et al. additionally speculated about possible use of the compounds in the treatment of a variety of diseases or conditions, including addictive disorders. The relevant descriptions in Adam et al., including the excerpts cited by the Examiner in rejecting the claimed invention in the Office Action, are reproduced below:

> "These compounds have been discovered to act as metabotropic glutamate receptor antagonists and accordingly are useful for the treatment of a range of neurological disorders, including psychosis, schizophrenia, Alzheimer's and other cognitive and memory disorders." [Adam et al., Col. 1, lines 54-58]
>
> "Further treatable indications are . . . as well as conditions which lead to glutamate-deficiency functions, such as e.g. muscle spasms, convulsions, migraine, urinary incontinence, nicotine addiction, opiate addiction, anxiety, vomiting, dyskinesia and depressions." [Adam et al., Col. 3, lines 16-24]

Other than these naked assertions, there were neither experimental data nor plausible substantiation anywhere in Adam et al. to prove or suggest that the compounds are indeed effective in treating drug dependence. More importantly, the pure speculation of Adam et al. is contradictory to the above-discussed results from actual scientific studies that were published in peer reviewed journals. Based on the speculative nature of the relevant assertions in Adam et al., as well as their contradiction to the consensus view of the skilled artisans in the relevant art, it is an inescapable conclusion that the prior art, including Adam et al., did not teach in any enabling manner treatment of drug dependence via mGluR2/3 antagonism.

C. **Examiner's assertion of presumption of validity of Adam et al.**

Appellants have previously pointed out that Adam et al., due to its absence of actual data or plausible substantiation, is not enabling with respect to the alleged

teaching of treating addictions via mGluR2/3 antagonism. In the Office Action, the Examiner dismissed Appellants' notion and asserted that "Adam et al. is a US Patent, which is believed to be enabled by its disclosure; . . . " (Office Action, page 8, last paragraph). With due respect, Appellants note that the Examiner's position is undoubtedly incorrect. This is because the presumption of validity of an issued U.S. patent only applies to the claimed invention (i.e., the claims) in the patent. The presumption certainly does not apply to any statement in the patent specification. It can by no means be applied to any unsubstantiated assertions or pure speculations imbedded in a patent specification, let alone speculations that are clearly contradictory to the consensus view of the skilled artisans that was founded on experimental results published in peer-reviewed scientific literatures.

From the foregoing, it can be concluded that the prior art did not teach treatment of drug dependence by antagonizing mGluR2 or mGluR3. Since the cited art did not teach each and every element of the presently appealed claims, a prima facie case of obviousness could not be established.

2. *No rationale for combining mGluR2/3 antagonism and mGluR5 antagonism*

Even assuming for the sake of argument that the prior art references cited by the Examiner did separately teach all the claimed elements, the rejected inventions are nonetheless non-obvious because no sound reasoning or rationale can be drawn from the prior art to combine the claimed elements. As noted by the Supreme Court, "a patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art. Although common sense directs one to look with care at a patent application that claims as innovation the combination of two known devices according to their established functions, it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed invention does." *KSR, KSR, 550 U.S. at ___, 82 USPQ2d at 1391.* The Board of Patent Appeals and

Interferences has also emphasized that "rejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness" (*In re Kahn*, 441 F. 3d 977, 988 (CA Fed. 2006) cited with approval in KSR) (emphasis added).

In the present case, the obviousness rejections rendered by the Examiner are improper because the Examiner did not provide any articulated reasoning or rationale for combining mGluR2/3 antagonism and mGluR5 antagonism in the treatment of addictions. In alleging that one would be motivated to combine a mGluR2/3 antagonist and a mGluR5 antagonist, the only reason advanced by the Examiner is that "they both treat addictive disorders" (Office Action, page 5, first paragraph). Other than this conclusory statement, the Examiner did not otherwise provide any scientific evidence or sound rationale to support the conclusion. More importantly, the Examiner's conclusion was also made in disregard to the fact that the relevant teachings available in the prior art all suggest that one would likely be taught away from such a combination.

A. **Counter-intuitive to target both mGluR2/3 and mGluR5**

It would have been counterintuitive to antagonize both a Group I mGluR (e.g., mGluR5) and a Group II mGluR (i.e., mGluR2 or mGluR3) in the treatment of addictions. This is because Group I glutamate receptors, such as mGlu5, are located postsynaptically and are excitatory receptors; thus, blockade of these receptors results in decreased glutamate signaling. On the other hand, Group II receptors, such as mGluR2/3, are located presynaptically and are inhibitory receptors; thus, their blockade will lead to increased release of glutamate and thus increased glutamate signaling. Such different biological activities of mGluR2/3 and mGluR5 were well known in the art, as evidenced by some of the pre-priority publications on record including, e.g., Kilbride et al., Eur. J. Pharmacol. 356:149, 1998 (attached as Ref. 5); Vignes et al.,

Neuropharmacol. 34:973-82, 1995 (attached as Ref. 6); and Schoepp, Neurochem. Int., 24:439, 1994 (attached as Ref. 7). The counter-intuitive nature of combining mGluR2/3 antagonism and mGluR5 antagonism due to the different activities of the receptors is explained in the attached copy of a declaration of Dr. Athina Markou under 37 CFR § 1.131 (attached as Ref. 8). The declaration was previously submitted by Appellants in the subject application. Dr. Athina Markou, a co-inventor of the claimed inventions, is an expert in the field of neuropharmacology and substance addictions. Dr. Markou stated in the declaration that "references from the literature suggesting that one should expect opposite neurochemical and behavioral effects of metabotropic glutamate 2/3 receptor (GluR2/3) antagonists and metabotropic glutamate receptor 5 (mGluR5) antagonists." The references referred to by Dr. Markou in the declaration include publications published before or around the same time of the priority date of the claimed inventions, e.g., Mills et al. (J. Neurochem. 79: 835-48, 2001; attached as Ref. 9), Xi et al. (J. Pharmacol. Exp. Ther. 300:162-71, 2002; attached as Ref. 10), Thomas et al. (Neuropharmacology 41: 523-7 2001; attached as Ref. 11) and de Novellis et al. (Eur. J. Pharmacol. 462: 73-81 2003; attached as Ref. 12).

The counter-intuitive nature to co-antagonize both Group I and Group II mGlu receptors suggest that a skilled artisan would not choose to combine the elements recited in the Appellants' claims. To the contrary, the artisan would probably be concerned that, due to the localization of these receptors in the synapse and their apparent opposing effects on postsynaptic glutamate signaling, co-administration of mGluR2/3 and mGluR5 antagonists would likely antagonize each other's activity to the extent that their effects are neutralized.

B. **The art taught away from co-antagonizing mGluR2/3 and mGluR5**

Citing Fundytus et al. (Brit. J. Pharmacol. 120:1015-20, 1997; attached as Ref. 4), the Examiner has previously rejected the claimed inventions as allegedly anticipated. The Examiner asserted that Fundytus et al. discloses treatment of morphine withdrawal

symptoms with α-methyl-4-carboxyphenylglycine (MCPG). The Examiner also noted that MCPG is an antagonist of both Group I glutamate receptors and Group II glutamate receptors. Appellants acknowledge that MCPG is a non-selective or dual antagonist of Group I and Group II mGluRs. However, the Examiner's interpretation of Fundytus et al. with respect to its relevance to the claimed inventions is clearly incorrect. As reiterated below, contrary to the Examiner's assertions, Fundytus et al. did not teach treatment of morphine withdrawal symptoms with MCPG. Instead, the experimental data reported in Fundytus et al. would likely teach away from Appellants' claimed inventions.

It is well known in the art that substance use/abuse and substance dependence are related but different concepts. Continued substance abuse can often lead to development of substance dependence (or addiction). Upon cessation of substance use (i.e., withdrawal), subjects already suffering from substance dependence will usually develop withdrawal symptoms. The difference between development of addiction in non-addictive subjects and withdrawal symptoms in addictive subjects is also clarified in Dr. Markou's declaration. Consistently, Appellants' claimed inventions relate to methods for treating addictive disorders in subjects that have already developed dependence on a given controlled substance (i.e., addiction). The inventions are directed to reducing, alleviating or eliminating withdrawal symptoms associated with cessation of substance use in subjects that have an existing addictive disorder. The claimed inventions are <u>not</u> directed to preventing the development of substance dependence in normal and healthy subjects who start using drugs but are not already suffering from substance dependence.

On the other hand, Fundytus et al. only showed that MCPG prevented <u>development</u> of drug dependence. Importantly, although the title and the abstract of Fundytus et al. might suggest otherwise, Fundytus et al. additionally reported that MCPG has <u>no</u> effect in the treatment of withdrawal symptoms in subjects which have already developed drug dependence. Specifically, Figure 1 of Fundytus et al., which

was relied on by the Examiner for the alleged teaching of treating drug dependence with MPCG, actually relates to a study of assessing the effect of various mGluR antagonists in the development of morphine dependence. The data were obtained from normal rats which were chronically administered morphine and at the same time treated with the mGluR antagonists. The goal of the study is clearly described in Fundytus et al., at page 1016, right column, last paragraph:

> Figure 1 illustrates the severity of abstinence symptoms during the 40 min withdrawal period in rats chronically infused with s.c. morphine and either vehicle, MCPG, MCCG or MAP4 i.c.v. This experiment was performed to determine if chronic blockade of mGluRs would attenuate the development of morphine dependence. [Emphasis added]

Results of the study is summarized in the below quoted passage of Fundytus et al. (at page 1016, left column, first paragraph, last 5 lines):

> In the present study, we showed that chronic non-selective antagonism of mGluRs with MCPG, and chronic selective antagonism of either group II or III mGluRs significantly attenuates the development of morphine dependence. [Emphasis added]

The results indicated that treatment with MCPG prevented the development of morphine dependence in normal, non-dependent rats. The lack of morphine dependence in the rats is evidenced by fewer signs of withdrawal when infusion of opiate is stopped. Unlike what the Examiner assumed, the data of Figure 1 did not show treatment of morphine dependent rats with MCPG, e.g., administration of MCPG to rats already having morphine dependence did not reduce symptoms upon opiate withdrawal.

More importantly, Fundytus et al. additionally examined whether, once rats were allowed to develop morphine dependence (i.e., "dependent rats" as noted in Fundytus et al.) and then opiate withdrawal symptoms expressed upon cessation of chronic

morphine administration, treatments with the same mGluR antagonists would have any effect on withdrawal symptoms. As indicated in Fundytus et al., the study was designed to "determine if acute blockade of mGluRs would decrease the expression of withdrawal symptoms once dependence had developed" (page 1017, left column, second to the last paragraph; emphasis added). Results from the study are shown in Figure 2 in Fundytus et al. As shown in the figure, none of the treatments (including treatment with MCPG) had any effect on withdrawal symptoms in morphine-dependent rats. Fundytus et al. expressly note that there is **"no difference between vehicle-treated rats and mGluR antagonist-treated rats"** (page 1018, left column, 2nd and 3rd paragraphs).

From the above clarifications, it is clear that Figure 1 of Fundytus et al. relates to normal rats (i.e., rats with no drug dependence) which were administered with MCPG together with morphine (to assess development of morphine dependence). The results indicate that simultaneous and chronic administration of morphine and the non-selective mGluR antagonist MCPG to healthy rats prevented the development of morphine dependence in the rats. However, the preventive effects in normal subjects evidenced by Figure 1 of Fundytus et al. are simply irrelevant to the claimed inventions. This is because the claimed inventions are not concerned with development of drug dependence, but are instead aimed at obtaining therapeutic effect in subjects who are already drug dependent. With a purpose of treating drug dependence in addictive subjects as presently claimed, the subjects certainly do not receive their medication together with the very drug on which they are already dependent (e.g., nicotine, cocaine or morphine) as in Figure 1 of Fundytus et al. Rather, the drug dependent subjects are administered only with the medication (i.e., the combination of an mGluR2/3 antagonist and an mGluR5 antagonist).

To summarize, Fundytus et al. taught that the dual mGluR antagonist MCPG PREVENTED the development of drug dependence (if co-administered TOGETHER with morphine) and the expression of the withdrawal signs in normal subjects (Fig. 1).

However, once dependence has already developed, the drug did NOT TREAT the withdrawal symptoms in the drug dependent subjects (Fig. 2). In other words, the data in Fundytus et al. that were relied on by the Examiner (i.e., Figure 1) are irrelevant to the claimed inventions. On the other hand and importantly, disclosures in Fundytus et al. that might be relevant to the claimed inventions (i.e., Figure 2) showed negative results, i.e., teaching away from the claimed invention.

3. *Surprising or unexpected results of the claimed inventions*

In addition to the prior art's lack of teaching of all the elements of the claimed inventions and also the prior art's likely teaching away from combining the recited elements, the non-obviousness nature of the claimed inventions is further demonstrated by the surprising or unexpected results disclosed in Appellants' application. For example, the subject specification disclosed that antagonizing mGluR2/3 (alone or in combination with mGluR5 antagonism) can produce beneficial effects in attenuating withdrawal symptoms (see, e.g., the summary at page 10 and Examples 3 at pages 64-79). These findings certainly represent surprising results that would not be expected from the prior art which reported that agonizing mGluR2/3 resulted in a reduction of withdrawal symptoms. Specifically, while the prior art showed that dual mGluR2/3 and mGluR5 antagonist MCPG had no effect in treating withdrawal symptoms in morphine-dependent rats, the subject specification taught that the combination of a mGluR5 antagonist (MPEP) and a mGluR2/3 antagonist (LY341495) was useful to treat established cocaine/nicotine dependence (see, e.g., Figures 14-16 and the discussions of Example 3.5 at page 76). Of particular importance is the finding by Appellants that the effect of the mGluR2/3 antagonist LY341495 on established nicotine dependence can be potentiated by co-administration of mGluR5 antagonist MPEP at a concentration where MPEP itself had no effect in treating addiction. Specifically, Figure 9C in the subject specification showed that MPEP at a dosage of 1 mg/kg had no effect in reducing nicotine or cocaine self-administration in dependent rats.

In contrast, as shown in Figures 14-15, such a MPEP dosage was effective in potentiating the inhibitory effects of the mGluR2/3 antagonist LY341495 on nicotine self-administration. The additive effects on inhibiting drug-taking behavior as illustrated by these data demonstrate that the combination of a mGluR2/3 antagonist and a mGluR5 antagonist as presently claimed is more effective than each of the two compounds alone in treating addictive disorders.

VIII. Summary of Arguments

To summarize, the presently appealed claims are non-obvious because the prior art did not teach each and every element recited in Appellants' claims. In addition, knowledge well known to the skilled artisans prior to Appellants' application would suggest that it is undesirable to combine the elements of Appellants' claims. Further, a reference cited by the Examiner as allegedly teaching Appellants' claimed inventions, i.e., Fundytus et al., could have actually led a skilled artisan away from the inventions. Moreover, the advantageous and surprising results demonstrated by the present inventors provide additional evidence that the presently claimed methods could not have been obvious. With due respect, Appellants note that the instant rejections are typical examples of "hindsight-based obviousness analysis." The alleged obviousness stems from nothing but the prohibited hindsight gleaned from Appellants' disclosure.

For all these reasons and the reasons already on record, Appellant respectfully requests that the Board of Patent Appeals and Interferences reverses the Examiner's rejections under 35 U.S.C. § 103(a) with respect to claims 1-3, 6, 7, 9, 16, 27, 28 and 32, and remands this application back to the Examiner for further examination.

If there are any fees associated with this Appeal Brief, please charge our Deposit Account No. 19-0962.

Respectfully submitted,

April 7, 2010

Date

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Claims Appendix

1. (previously presented) A method for treating drug dependence in a subject, comprising administering to a subject with drug dependence an effective amount of (a) a first antagonist which modulates metabotropic glutamate receptor 2 and/or metabotropic glutamate receptor 3, and (b) a second antagonist which modulates metabotropic glutamate receptor 5, thereby treating the disorder; wherein the drug dependence is selected from the group consisting of nicotine addiction, alcohol addiction, opiate addiction, amphetamine addiction, cocaine addiction, and methamphetamine addiction.

2. (previously presented) A method for treating drug dependence in a subject, comprising administering to a subject with drug dependence an effective amount of (a) a first antagonist which modulates metabotropic glutamate receptor 2, and (b) a second antagonist which modulates metabotropic glutamate receptor 5, thereby treating the disorder; wherein the drug dependence is selected from the group consisting of nicotine addiction, alcohol addiction, opiate addiction, amphetamine addiction, cocaine addiction, and methamphetamine addiction.

3. (previously presented) A method for treating drug dependence in a subject, comprising administering to a subject with drug dependence an effective amount (a) a

first antagonist which modulates metabotropic glutamate receptor 3 and (b) a second antagonist which modulates metabotropic glutamate receptor 5, thereby treating the disorder; wherein the drug dependence is selected from the group consisting of nicotine addiction, alcohol addiction, opiate addiction, amphetamine addiction, cocaine addiction, and methamphetamine addiction.

4-5. (canceled)

6. (previously presented) The method of claim 1, wherein the drug dependence is nicotine addiction.

7. (previously presented) The method of claim 1, wherein the drug dependence is cocaine addiction.

8. (canceled)

9. (previously presented) The method according to claim 1, wherein the antagonist which modulates metabotropic glutamate receptor 5 is 2-methyl-6-(phenylethynyl)-pyridine, and the antagonist which modulates metabotropic glutamate receptor 2 and/or metabotropic glutamate receptor 3 is 2S-2-amino-2-(1S,2S-2-carboxycyclopropan-1-yl)-3-(xanth-9-yl)propionic acid .

10. (withdrawn) A combination comprising (a) at least a first active ingredient selected from a metabotropic glutamate receptor 2 antagonist and a metabotropic glutamate receptor 3 antagonist, and (b) at least a second active ingredient being a metabotropic glutamate receptor 5 antagonist, in which the active ingredients are present in each case in free form or in the form of a pharmaceutically acceptable salt, and optionally at least one pharmaceutically acceptable carrier; for simultaneous, separate or sequential use.

11-13. (canceled)

14. (withdrawn) The combination according to claim 10 which is a combined preparation or a pharmaceutical composition.

15. (withdrawn) The combination according to claim 10 for simultaneous, separate or sequential use in the treatment of an addictive disorder or depression.

16. (previously presented) A method of treating a warm-blooded animal having an addictive disorder comprising administering to the animal a combination according to claim 10 in a quantity which is jointly therapeutically effective against an addictive disorder and in which the compounds can also be present in the form of their

pharmaceutically acceptable salts; wherein the addictive disorder is selected from the group consisting of nicotine addiction, alcohol addiction, opiate addiction, amphetamine addiction, cocaine addiction, and methamphetamine addiction.

17. (withdrawn) A pharmaceutical composition comprising a quantity, which is jointly therapeutically effective against an addictive disorder or depression, of a pharmaceutical combination according to claim 10 and at least one pharmaceutically acceptable carrier.

18. (canceled)

19. (withdrawn) A commercial package comprising a combination according to claim 10 together with instructions for simultaneous, separate or sequential use thereof in the treatment of an addictive disorder.

20-26. (canceled)

27. (previously presented) A method for treating an addictive disorder, comprising: a) administering to a subject in need thereof, an effective amount of a first antagonist that modulates mGluR5 during a first time period, wherein the first time period is a time period wherein the subject expects to be in an environment wherein, or exposed to

stimuli in the presence of which, the subject habitually uses an addictive substance; and b) administering a second antagonist that modulates mGluR2 and/or 3 during a second time period, wherein the second time period is a time period wherein the subject is suffering from withdrawal; wherein the addictive disorder is selected from the group consisting of nicotine addiction, alcohol addiction, opiate addiction, amphetamine addiction, cocaine addiction, and methamphetamine addiction.

28. (previously presented) The method of claim 27, wherein the antagonist that modulates mGluR5 is 2-methyl-6-(phenylethynyl)-pyridine and the antagonist that modulates mGluR2 and/or 3 is 2S-2-amino-2-(1S,2S-2-carboxycyclopropan-1-yl)-3-(xanth-9-yl)propionic acid.

29-31. (canceled)

32. (previously presented) The method of claim 1, wherein the first antagonist and the second antagonist are administered to the subject sequentially or simultaneously.

33. (canceled)

Evidence Appendix

Copies of documents relied upon by Appellants are enclosed herewith. Dates of entry of these references into record are listed below.

I. Documents cited by the Examiner in the Office Action dated May 3, 2007

1. Adam et al. (U.S. Patent No. 6,406,094);
2. Corsi et al. (U.S. Application 2003/0195139);
3. Chiamulera et al. (Nat Neurosci. 4:873-874, 2001); and
4. Fundytus et al. (Brit. J. Pharmacol. 120:1015-20, 1997).

II. Documents submitted by Appellants on September 4, 2007 and acknowledged by the Examiner in the Office Action dated November 13, 2007.

5. Kilbride et al. (Eur. J. Pharmacol. 356:149, 1998);
6. Vignes et al. (Neuropharmacol. 34:973-82, 1995); and
7. Schoepp (Neurochem. Int., 24:439, 1994).

III. Documents submitted by Appellants on September 23, 2008 and acknowledged by the Examiner in the Office Action dated January 8, 2009

8. Declaration of Dr. Athina Markou under 37 CFR § 1.131;
9. Mills et al. (J. Neurochem. 79: 835-48, 2001);
10. Xi et al. (J. Pharmacol. Exp. Ther. 300:162-71, 2002); and
11. Thomas et al. (Neuropharmacology 41: 523-7 2001).
12. de Novellis et al. (Eur J Pharmacol 462: 73-81 2003):

IV. Documents submitted by Appellants on August 27, 2009 and acknowledged by the Examiner in the Office Action dated December 15, 2009

13. Helton et al. (Neuropharmacol. 36:1522-6, 1997);
14. Vandergriff and Rasmussen (Neuropharmacol. 38:217-22, 1999);
15. Fundytus and Coderre (Brit. J. Pharmacol. 121:511-4, 1997); and
16. Kenny et al. (J. Pharmacol. Exp. Ther. 306:1068-76, 2003).

V. 17. Final Office Action dated 12/15/2009

Related Proceedings Appendix

None.

for appeal brief USSN 10/527,525

Ref. 1

US006407094B1

(12) **United States Patent**
Adam et al.

(10) **Patent No.: US 6,407,094 B1**
(45) **Date of Patent: Jun. 18, 2002**

(54) **GLUTAMATE RECEPTOR ANTAGONISTS**

(75) Inventors: **Geo Adam**, Schopfheim (DE); **Alexander Alanine**, Riedisheim (FR); **Erwin Goetschi**, Reinach (CH); **Vincent Mutel**, Mulhouse (FR); **Thomas Johannes Woltering**, Weil am Rhein (DE)

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/687,240**

(22) Filed: **Oct. 13, 2000**

(30) **Foreign Application Priority Data**

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(51) **Int. Cl.**[7] **C07D 243/12**; C07D 409/04; C07D 405/04; C07D 417/04; A61K 31/55

(52) **U.S. Cl.** **514/221**; 540/517

(58) **Field of Search** 540/517; 514/221

(56) **References Cited**

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WO	WO 99/26927	6/1999

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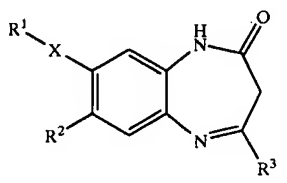
(57) **ABSTRACT**

The present invention relates to compounds of with a base structure of formula 1

I



The compounds of formula I are shown to have activity as metabotropic glutamate receptor antagonists.

7 Claims, No Drawings

GLUTAMATE RECEPTOR ANTAGONISTS

BACKGROUND OF THE INVENTION

In the central nervous system (CNS) the transmission of stimuli takes place by the interaction of a neurotransmitter, which is sent out by a neuron, with a neuroreceptor.

L-glutamic acid, the most commonly occurring neurotransmitter in the CNS, plays a critical role in a large number of physiological processes. The glutamate-dependent stimulus receptors are divided into two main groups. The first main group forms ligand-controlled ion channels. The metabotropic glutamate receptors (mGluR) form the second main group and, furthermore, belong to the family of G-protein-coupled receptors.

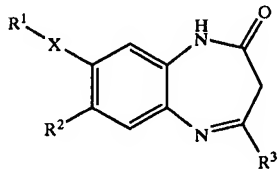
At present, eight different members of these mGluR are known and of these some even have sub-types. On the basis of structural parameters, the different influences on the synthesis of secondary metabolites and the different affinity to low-molecular weight chemical compounds, these eight receptors can be sub-divided into three sub-groups: mGluR1 and mGluR5 belong to group I, mGluR2 and mGluR3 belong to group II and mGluR4, mGluR6, mGluR7 and mGluR8 belong to group III.

Ligands of metabotropic glutamate receptors belonging to the group II can be used for the treatment or prevention of acute and/or chronic neurological disorders such as psychosis, schizophrenia, Alzheimer's disease, cognitive disorders and memory deficits.

SUMMARY OF THE INVENTION

The present invention relates to compounds of the general formula I

I



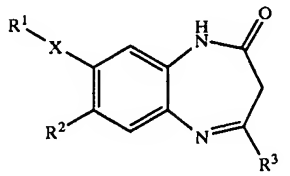
These compounds have been discovered to act as metabotropic glutamate receptor antagonists and accordingly are useful for the treatment of a range of neurological disorders, including psychosis, schizophrenia, Alzheimer's and other cognitive and memory disorders.

Objects of the present invention are compounds of formula I and their pharmaceutically acceptable salts per se and as pharmaceutically active substances, their manufacture, medicaments based on a compound in accordance with the invention and their production, as well as the use of the compounds in accordance with the invention in the control or prevention of neurologial disorders, and, respectively, for the production of corresponding medicaments.

DETAILED DESCRIPTION

The present invention relates to compounds of formula I

I



wherein

X is a single bond or an ethynediyl group, wherein,

In case X is a single bond, R^1 is halogen or phenyl which is optionally substituted with halogen, lower alkyl, halo-lower alkyl, lower alkoxy, halo-lower alkoxy, or cyano;

In case X is an ethynediyl group, R^1 is phenyl, optionally substituted with halogen, lower alkyl, halo-lower alkyl, lower cycloalkyl, lower alkoxy or halo-lower alkoxy;

R^2 is halogen; hydroxy; lower alkyl; lower halo-alkyl; lower alkoxy; hydroxymethyl; hydroxyethoxy; lower alkoxy-(ethoxy)$_n$ (n=1 to 4); lower alkoxymethyl; cyanomethoxy; morpholine-4-yl; thiomorpholine-4-yl; 1-oxothiomorpholine-4-yl; 1,1-dioxothiomorpholine-4-yl; 4-oxo-piperidine-1-yl; 4-alkoxy-piperidine-1-yl; 4-hydroxy-piperidine-1-yl; 4-hydroxyethoxy-piperidine-1-yl; 4-lower alkyl-piperazine-1-yl; alkoxycarbonyl; 2-dialkylamino-ethylsulfanyl-; N,N-bis lower alkylamino lower alkyl; carbamoylmethyl; alkylsulfonyl; lower alkoxycarbonyl-lower alkyl; alkylcarboxy-lower alkyl; carboxy-lower alkyl; alkoxycarbonylmethylsulfanyl; carboxymethylsulfanyl; 1,4-dioxa-8-aza-spiro[4.5]dec-8-yl; carboxy-lower alkoxy; cyano-lower alkyl; 2,3-dihydroxy-lower alkoxy; carbamoylmethoxy; 2-oxo-[1,3]-dioxolan-4-yl-lower alkoxy; (2-hydroxy-lower alkyl)-lower alkyl amino; hydroxycarbamoyl-lower alkoxy; 2,2-dimethyl-tetrahydro-[1,3]dioxolo[4,5c]-pyrrol-5-yl; lower alkoxy-carbamoyl-lower alkoxy; 3R-hydroxy-pyrrolidin-1-yl; 3,4-dihydroxy-pyrrolidin-1-yl; 2-oxo-oxazolidin-3-yl; lower alkyl-carbamoylmethoxy; or aminocarbamoyl-lower alkoxy;

R^3 is a 5 or 6 membered aryl or heteroaryl which are optionally substituted by halogen; cyano; nitro; azido; hydroxy; carboxy; morpholine-4-carbonyl; carbamoyl; thiocarbamoyl; N-hydroxycarbamoyl; trimethylsilyl-ethynyl; or lower alkyl; lower alkoxy; halo-lower alkyl; 4-lower alkyl-piperazine-1-carbonyl; lower alkylcarbamoyl which are optionally substituted by amino, lower alkylamino, acylamino, oxo, hydroxy, lower alkoxy, lower alkylthio, or carboxy which is optionally esterified or amidated; or an optionally substituted five-membered aromatic heterocycle, which may be optionally substituted by amino, lower alkylamino, acylamino, oxo, hydroxy, lower alkoxy, lower alkylthio, or carboxy which is optionally esterified or amidated, or lower alkyl which is optionally substituted by halogen, amino, lower alkylamino, acylamino, hydroxy, lower alkoxy, lower alkylthio, acyloxy, lower alkenoyl, lower alkylsulfinyl, lower alkylsulfonyl, cycloalkylsulfinyl, cycloalkylsulfonyl, hydroxyimino, alkoxyimino, carboxy which is optionally esterified or amidated, lower alkenyl, oxo, cyano, carbamoyloxy, sulfamoyl which is optionally substituted by lower

alkyl, or amidino which is optionally substituted by lower alkyl, —C(NRR')=NR" (where R, R' and R" are hydrogen or lower alkyl)

and their pharmaceutically acceptable addition salts.

It has surprisingly been found that the compounds of formula I are metabotropic glutamate receptor antagonists. Compounds of formula I are distinguished by valuable therapeutic properties.

The compounds of the present invention can be used for the treatment or prevention of acute and/or chronic neurological disorders such as psychosis, schizophrenia, Alzheimer's disease, cognitive disorders and memory deficits.

Other treatable indications in this connection are restricted brain function caused by bypass operations or transplants, poor blood supply to the brain, spinal cord injuries, head injuries, hypoxia caused by pregnancy, cardiac arrest and hypoglycaemia. Further treatable indications are chronic and acute pain, Huntington's chorea, amyotrophic lateral scherosis (ALS), dementia caused by AIDS, eye injuries, retinopathy, indiopathic parkinsonism or parkinsonism caused by medicaments as well as conditions which lead tn glutamate-deficiency functions, such as e.g. muscle spasms, convulsions, migraine, urinary incontinence, nicotine addiction, opiate addiction, anxiety, vomiting, dyskinesia and depressions.

Objects of the present invention are compounds of formula I and their pharmaceutically acceptable salts per se and as pharmaceutically active substances, their manufacture, medicaments based on a compound in accordance with the invention and their production, as well as the use of the compounds in accordance with the invention in the control or prevention of illness of the aforementioned kind, and, respectively, for the production of corresponding medicaments.

Preferred compounds of formula I in the scope of the present invention are those in which R^3 is phenyl substituted in meta position by cyano; halogen; or imidazolyl which is optionally substituted by lower alky or methylsulfanyl; 1,2,3-triazolyl; 1,2,4-triazolyl; or isoxazolyl which is optionally substituted by lower alkyl.

The following are examples of such compounds:

3-(8-Chloro-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo [b][1,4]diazepin-2-yl)-benzonitrile

3-[8-(4-Methyl-piperazin-1-yl)-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile;

3-(8-Chloro-4-oxo-7-phenyl-4,5-dihydro-3H-benzo[b][1,4] diazepin-2-yl)-benzonitrile;

[4-(3-Cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-ylsulfanyl]-acetic acid methyl ester;

2-[4-(3-Cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yl]-acetamide;

3-(8-Methoxy-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2yl)-benzonitrile

3-(8-Cyanomethyl-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-benzonitrile;

4-(3-Iodo-phenyl)-7-(2-methoxy-ethoxy)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

4-(3-Imidazol-1-yl-phenyl)-7-(2-methoxy-ethoxy)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

[RS]-3-[4-Oxo-8-(2-oxo-[1,3]dioxolan-4-ylmethoxy)-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile;

7-Hydroxymethyl-4-(3-imidazol-1-yl-phenyl)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

[4-(3-Imidazol-1-yl-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yloxy]-acetonitrile;

8-(4-Fluoro-phenylethynyl)-7-hydroxymethyl-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4] diazepin-2-one;

7-(2-Hydroxy-ethoxy)-4-(3-imidazol-1-yl-phenyl)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

8-(4-Fluoro-phenyl)-7-[4-(2-hydroxy-ethoxy)-piperidin-1-yl]-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

8-(4-Fluoro-phenyl)-7-hydroxy-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

8-(2-Fluoro-phenyl)-7-methoxy-4-[3-(2-methyl-imidazol-1-yl)-phenyl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

8-(2-Fluoro-phenyl)-7-hydroxy-4-(3-[1,2,3]triazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

8-(2-Fluoro-phenyl)-7-hydroxy-4-[3-(2-methyl-imidazol-1-yl)-phenyl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

8-(2-Fluoro-phenyl)-7-hydroxy-4-[3-(2-methylsulfanyl-imidazol-1-yl)-phenyl]-1,3-dihydro-benzo[b][1,4] diazepin-2-one;

8-(2,5-Difluoro-phenyl)-7-methoxy-4-(3-[1,2,3]triazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

8-(2-Fluoro-phenyl)-7-hydroxy-4-[3-(3-methyl-isoxazol-5-yl)-phenyl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

3-[7-(2,5-Difluoro-phenyl)-8-hydroxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile;

8-(4-Fluoro-phenylethynyl)-7-hydroxy-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one; and

8-(4-Fluoro-phenylethynyl)-7-hydroxy-4-(3-[1,2,3]triazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one.

Compounds of formula 1, wherein R^3 is thiophenyl, preferably thiophen-2-yl, which is optionally substituted by cyano or halogen; or R^3 is pyridinyl, preferably pyridin-4-yl, which is optionally substituted in 2-position by cyano or halogen, or wherein R^3 is thiazolyl which is optionally substituted in 2-position with imidazolyl or 4-methylimidazolyl, are also preferred.

The following compounds are particularly preferred:

5-[7-(2-Fluoro-phenyl)-8-methoxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-thiophene-2-carbonitrile;

2-[7-(2-Fluoro-phenyl)-8-hydroxy-4-oxo-4,5-dihydro-1H-benzo[b][1,4]diazepin-2-yl]-thiophene-3-carbonitrile;

4-[7-(2-Fluoro-phenyl)-8-methoxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-pyridine-2-carbonitrile;

4-[7-(4-Fluoro-phenyl)-8-hydroxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-pyridine-2-carbonitrile;

4-[7-(2-Fluoro-phenyl)-8-hydroxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-pyridine-2-carbonitrile; and

8-(2-Fluoro-phenyl)-4-[2-(4-methyl-imidazol-1-yl)-thiazol-4-yl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one.

All tautomeric forms of the compounds of the invention are also embraced herewith.

The term "lower alkyl" used in the present description denotes straight-chain or branched saturated hydrocarbon residues with 1–7 carbon atoms, preferably with 1–4 carbon atoms, such as methyl, ethyl, n-propyl, i-propyl and the like.

The term "lower cycloalkyl" used in the present description denotes cyclic saturated hydrocarbon residues with 3–5 carbon atoms, preferably with 3 carbon atoms, such as cyclopropyl.

The term "lower alkoxy" denotes a lower alkyl residue in the sense of the foregoing definition bonded via an oxygen atom.

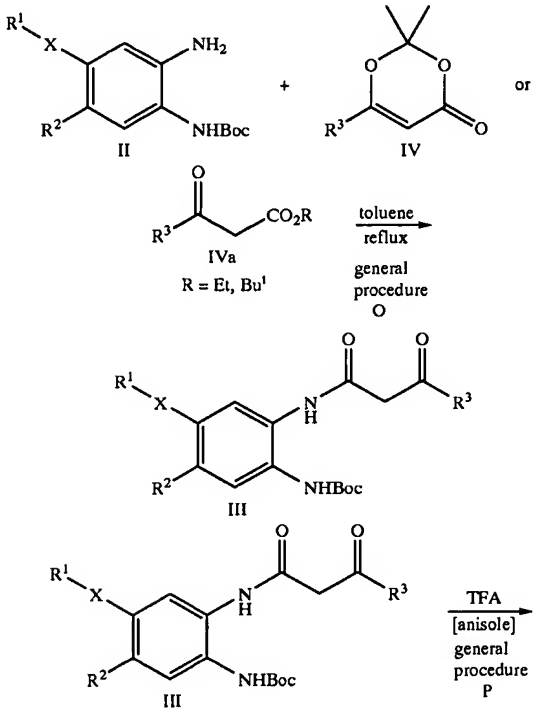
The term "halogen" embraces fluorine, chlorine, bromine and iodine.

The term "5 or 6 membered aryl or heteroaryl" embraces phenyl, thiophenyl, pyridine, partially hydrated pyridine.

The expression "five-membered aromatic heterocycle" embraces, furan, thiazol, imidazol, pyrazol, 1,3-thiazol, 1,3-oxazol, 1,2-oxazol, 1,2-thiazol, 1,2,3-triazol, 1,2,4-triazol, 1,2,4-oxadiazol, 1,2,3-oxadiazol, 1,2,4-thiadiazol, 1,2,3-thiadiazol and tetrazol.

The compounds of formula I and their pharmaceutically acceptable salts can be manufactured according to the following methods:

Scheme A



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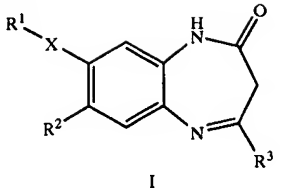


I

According to scheme A, compounds of formula I, in which X, R^1, R^2 and R^3 are as described above, can be prepared from compounds of formula II via an acylation-deprotection-cyclization sequence:

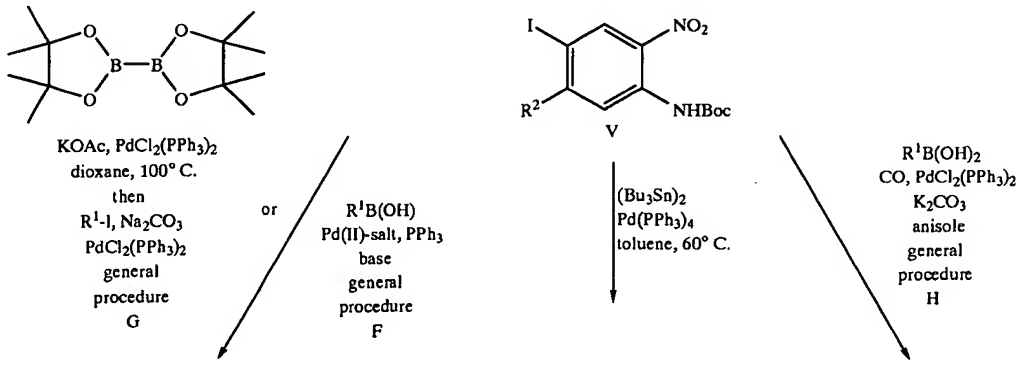
For example reacting compounds of formula II with a dioxinone IV, in which R^3 is as described above, in an inert solvent such as toluene or xylene at elevated temperatures, preferably between 80° C. and 160° C. gives rise to compounds of formula III.

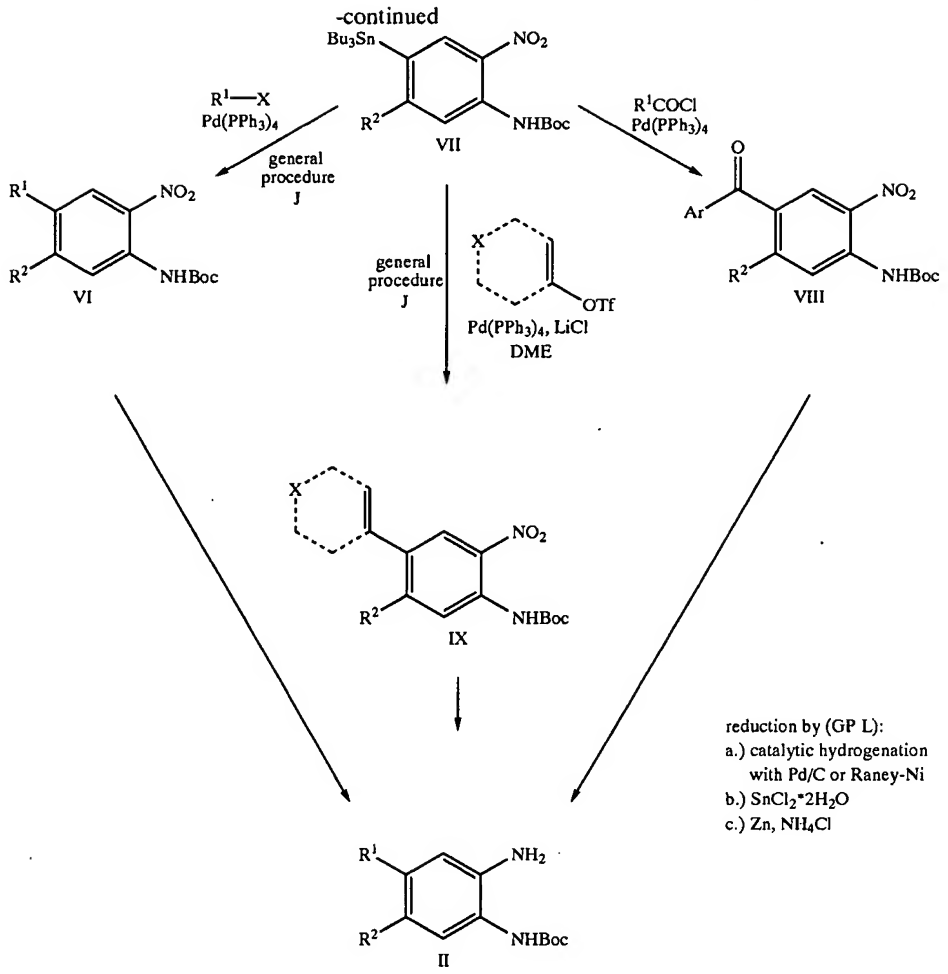
Alternatively, compounds of formula III can also be prepared by for example reaction of a compound of formula II with a β-ketoester (formula IVa), in which R^3 is as described above using the same conditions as described for the reaction with the dioxinones.

Afterwards, cleaving the BOC protecting group in compounds of formula III and concomitant cyclization of the deprotected compound yields the desired compounds of formula I. Any other suitable amino protecting group, such as e.g. Fmoc or benzyloxycarbonyl (Z), can be alternatively used instead of the BOC group.

The deprotection-cyclization step can be carried out by treating the compounds of formula III with for example a Bronsted acid such as trifluoroacetic acid in an inert solvent such as dichloromethane (DCM). The reaction is preferably carried out at temperatures between 0° C. and 50° C. It may be advantageous to use also anisole or 1,3-dimethoxybenzene as a carbocation scavenger in the reaction mixture.

Scheme B

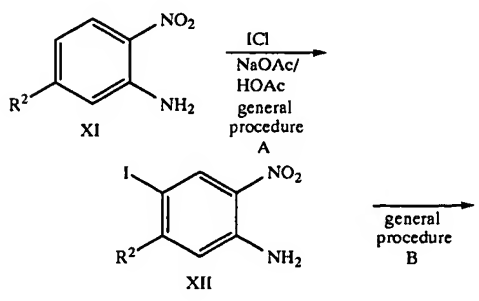




According to scheme B, compounds of formula II in which R^1 is as described above for compounds where X is a single bond and R^2 is as described above, can be prepared by different routes depending on the nature of R^2 from the iodo-compounds of formula V, in which R^2 is as described above. As shown in scheme B, the key steps are coupling reactions of Suzuki-and Stille-type in presence or absence of carbonmonoxide. The exact conditions for the respective compounds of formula II can be found in the experimental part.

Compounds of formula V, in which R^2 is as described above, can be prepared by different routes depending on the individual residue R^2:

Scheme C



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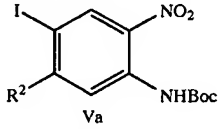


Va

GP B, method a: diphosgene, EtOAc, 77° C.; then t-BuOH
GP B, method b: Boc_2O, Cs_2CO_3, 2-butanone, 52° C.
GP B, method c: i) Boc_2O, DMAP, THF; ii) TFA, DCM, 0° C.

As shown in scheme C, compounds of formula Va, in which R^2 is lower alkyl, halogen or alkoxycarbonyl, can be prepared from the known compounds of formula XI by iodination and subsequent protection of the synthetic intermediates with formula XII.

The iodination step can be carried out by for example using iodine monochloride in acetic acid in the presence of sodium acetate. The reaction can be for example carried out at temperatures between 20° C. and 80° C.

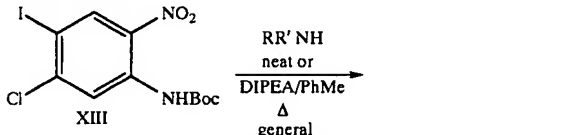
The protection of the amino function can be achieved by for example reacting compounds of formula XII with di-tert.-butyl-carbonate in the presence of a base such as cesium carbonate. The reaction can be carried out in polar solvents such as acetone or butanone and the like at temperatures between 20° C. and 60° C.

As shown in scheme D, compounds of formula Vb and Vc, in which R^2 is attached via a sulfur- or nitrogen-atom (R^2 represents for example morpholin-4-yl; thiomorpholino-

4-yl; dialkylamino; carboxymethylsulfanyl etc), respectively, can be prepared from the intermediate XIII by a nucleophilic substitution reaction with the respective amines or mercaptanes in the presence of a suitable base.

Scheme D

Nitrogen nucleophiles



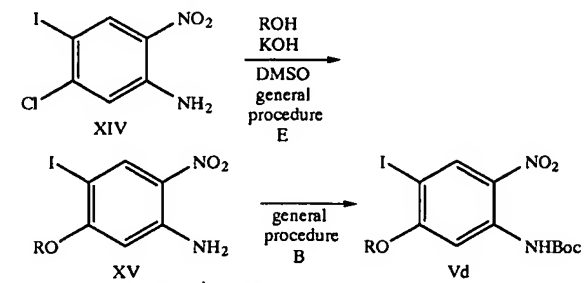
Sulfur nucleophiles

The reaction is preferably carried out in a polar, aprotic solvent such as dimethyl formamide, N-methyl-pyrrolidone or dimethyl sulfoxide and the like. The base can be selected from the sterically hindered amines such as Hünig's base, alkoxides such as sodium methoxide and tert.-butoxide, or hydrides such as sodium hydride. The reaction can be performed at temperatures between 20° C. and 110° C., depending on the individual compounds to be synthesized.

Compounds of formula Vd in which R^2 is attached via an oxygen atom (R^2 represents for example lower alkoxy, lower halo-alkoxy, lower cyclo-alkoxy, lower alkoxy-lower alkoxy; etc.) can be prepared as for example shown in scheme E:

Scheme E

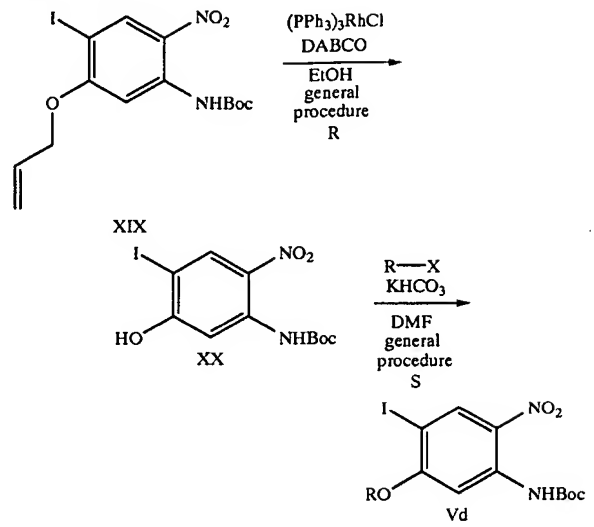
Oxygen nucleophiles



GP B, method a: diphosgene, EtOAc, 77° C.; then t-BuOH
GP B, method b: Boc_2O, Cs_2CO_3, 2-butanone, 52° C.
GP B, method c: i) Boc_2O, DMAP, THF; ii) TFA, DCM, 0° C.

-continued

Oxygen alkylation



by a nucleophilic aromatic substitution reaction with the respective alcohol in the presence of a suitable base and subsequent protection of the amino function. The base can be selected from the class of Bronsted bases such as potassium hydroxide and the like. The reaction is preferably carried out in a polar, aprotic solvent such as dimethyl formamide, N-methyl-pyrrolidone or dimethyl sulfoxide and the like at temperatures between 20° C. and 100° C.

The protection of the amino function can be achieved by for example reacting compounds of formula XV with di-tert.-butoxy carbonate in the presence of a base such as cesium carbonate. The reaction can be carried out in polar solvents such as acetone or butanone and the like at temperatures between 20° C. and 60° C.

Another method to achieve this protection step is to transform first the amino function in a compound with formula XV into an isocyanate by reaction with phosgene or a phosgene equivalent in the presence of a suitable base, which is then treated with tert.-butyl-alcohol to give the desired compounds of formula Vd.

Another suitable method to achieve this protection step is to transform first the amino function in a compound with formula XV into the corresponding di-Boc compound by reaction with excess di-tert.-butoxy carbonate in the presence of 4-dimethylaminopyridine (DMAP), which is then treated with 2eq. TFA in dichloromethane to give the desired compounds of formula Vd.

This reversal of steps, i.e. performing first the nucleophilic aromatic substitution on the key intermediate XIV and second protection of the amino-function as shown in synthetic scheme E can also be applied to those compounds with the formula Vb and Vc (synthetic scheme D).

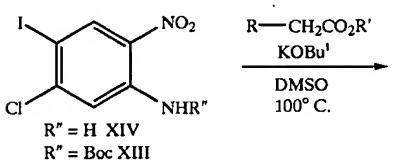
Yet another method of preparing compounds of the formula Vd is using the O-allyl compound XIX and perfoming a deallylation-alkylation sequence as outlined in scheme E. The deallylation is preferably carried out by transition-metal catalyzed isomerisation, e.g. in the presence of Rhodium(I)-salts like for example Wilkinson's catalyst [$(PPh_3)_3RhCl$] or Palladium(II)-salts such as [$(PPh_3)_2PdCl_2$], followed by aqueous acid hydrolysis of the resulting vinyl ether. An example for this procedure can be found in *J. Org. Chem.* 1973, 38, 3224. The alkylation of the phenol XX to the desired compound of the formula Vd can be carried out with electrophilic reagents of the formula R—X, in which R has

the meaning of lower alkyl, lower alkenyl, alkyl acetate or benzyl and X represents a leaving group, for example iodide, bromide, methanesulfonate or tolylsulfonate, in a suitable solvent in the presence of a base. The reaction is preferably carried out in polar, aprotic solvents, for example chlorinated solvents such as dichloromethane, chloroform or dichloroethane, or amides, for example dimethylformamide, dimethylacetamide and N-methyl-pyrrolidone, or sulfoxides, for example dimethyl sulfoxide. The base can be selected from the sterically hindered amines such as Hünig's base, alkoxides such as sodium methoxide and tert.-butoxide, hydrides such as sodium hydride, hydroxides such as potassium hydroxide, carbonates such as potassium carbonate or hydrogen carbonates such as potassium hydrogen carbonate. The reaction can be performed at temperatures between -20° C. and 80° C., depending on the individual compounds to be synthesized. For the synthesis of an O-tert.-butyl compound with the formula Vd the phenol XX can be treated with DMF-di-tert.-butylacetal in toluene or benzene at 80° C. as described in *Synthesis* 1983, 135.

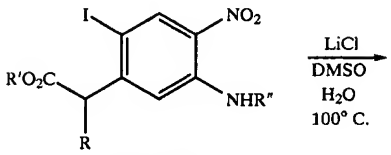
According to synthetic scheme F,

Scheme F

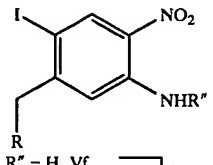
Carbon nucleophiles



R" = H XIV
R" = Boc XIII

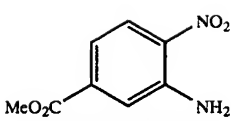


R" = H XVII
R" = Boc XVI



R" = H Vf
R" = Boc Ve — GP B

Cl moiety



Methyl 3-amino-4-nitrobenzoate

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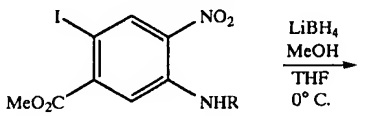
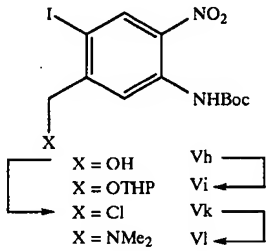


R = H XXI
R = Boc Vg — GP B

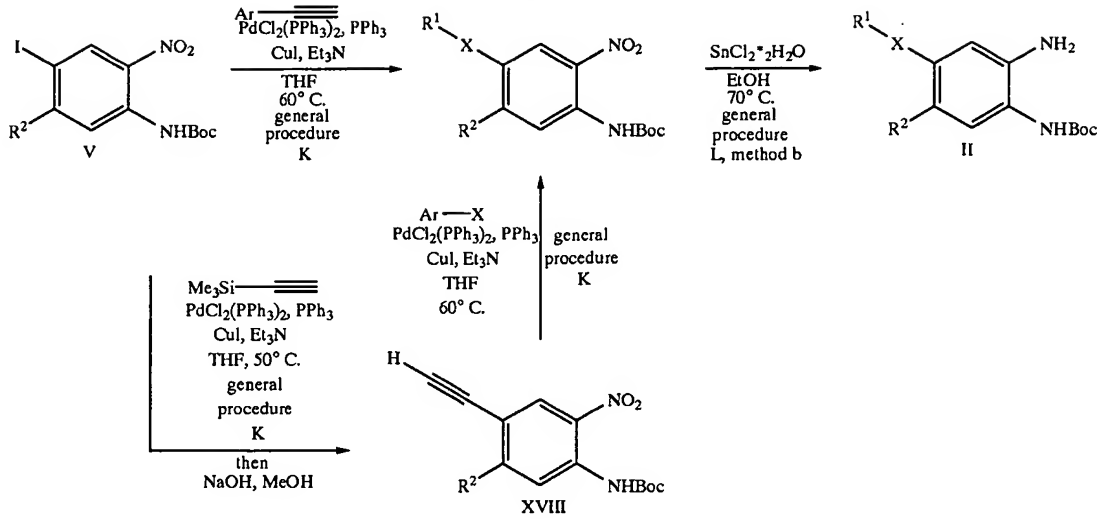


compounds of formula Ve and Vf in which R^2 is attached via a carbon atom (R^2 represents for example lower alkyloxy-carbonyl-methyl; cyano-methyl, etc.) can be prepared from compound XIII or XIV by for example reaction with a malonic acid ester or -half-ester in the presence of a base followed by the removal of one of the alkyl carboxylates via decarboxylation. The exact reaction conditions vary with the identity of the individual compounds and are described in the examples.

The key intermediates XIII and XIV can be prepared as already described in scheme C.

For the one-carbon-moiety bearing compounds of the formula Vh to VI, the synthesis starts from known methyl 3-amino-4-nitrobenzoate. Standard iodination as described in synthetic scheme C leads to the iodide XXI, which in turn can be protected with the Boc-group. The reduction of the methyl ester can for example be performed by treatment with lithium borohydride, sodium borohydride or diisobutylaluminumhydride in an aprotic solvent like for example THF, ether or toluene. The presence of an alcohol such as methanol, ethanol or isopropanol can be advantegous. The reduction is preferably carried out at temperatures between -20° C. and 0° C. Further functionalization, like for example conversion into a chloride (Vk), of the resulting benzylic alcohol Vh follows standard procedures known to someone skilled in the art. The exact reaction conditions vary with the identity of the individual compounds and are described in the examples.

Scheme G



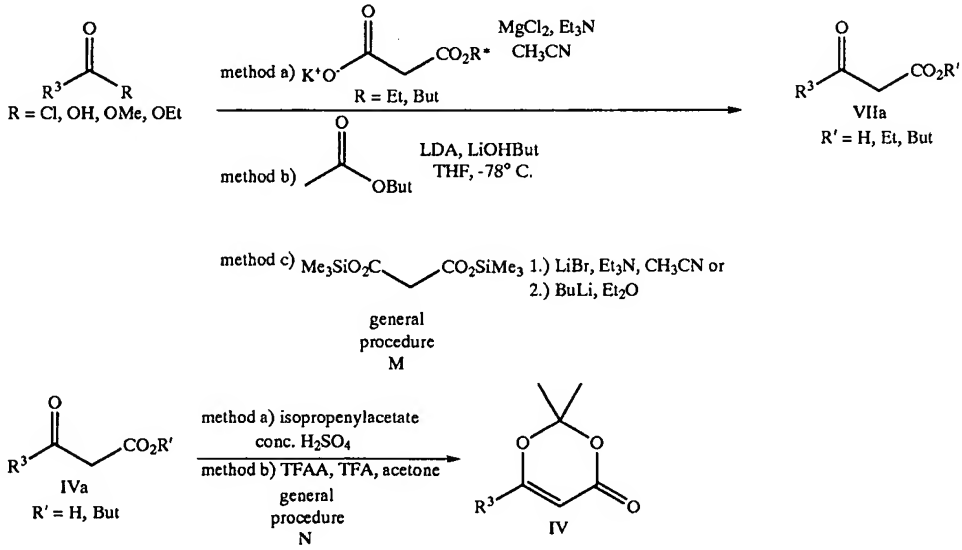
According to scheme G, compounds of formula II in which R^1 is as described above for compounds where X is an ethynediyl group can be prepared by different routes from the iodo-compounds V, depending on the nature of R^1 and R^2As shown in scheme G, the transformation can for example be carried out

a) by directly attaching the R^1-alkynediyl-substituent to a compound of formula V via a Sonogashira-type coupling followed by the reduction of the nitro group or

b) by two stepwise Sonogashira-type couplings, in which first trimethylsilyl-acetylene is coupled to a compound of formula V to yield, after deprotection with sodium hydroxide in methanol, the intermediate XVIII which then can be transformed via a second Sonogashira-type coupling with the appropriate reactant R^1—I, R^1—Br or R^1—$OSO_2CF_3$and reduction of the nitro group to the desired compounds of formula II.

The exact conditions for the respective compounds can be found in the experimental part.

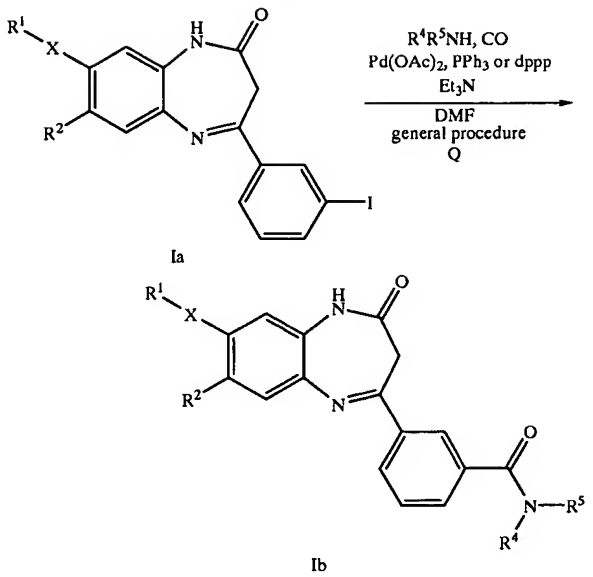
Scheme H



According to Scheme H, the dioxinones and β-keto esters building blocks with the formula IV and IVa can be prepared by methods known to someone skilled in the art from the corresponding carboxylic acid derivatives R^3—COR, i.e. free acids, methyl or ethyl esters and acid chlorides. The exact conditions for the corresponding compounds can be found in the experimental part.

Another synthetic route to prepare compounds of formula I in which R^1, $R^2$and X have the meaning as described above and $R^3$is a carbamide of formula $C(O)NR^4R^5$, in which R^4 and R^5 is hydrogen, lower alkyl or R^4 and R^5 together form a morpholino-residue or a N-methyl-piperazine, is outlined in scheme I:

Scheme I



Ia

Ib

The exact conditions for the respective compounds can be found in the experimental part.

The pharmaceutically acceptable salts can be manufactured readily according to methods known per se and taking into consideration the nature of the compound to be converted into a salt. Inorganic or organic acids such as, for example, hydrochloric acid, hydrobromic acid, sulphuric acid, nitric acid, phosphoric acid or citric acid, formic acid, fumaric acid, maleic acid, acetic acid, succinic acid, tartaric acid, methanesulphonic acid, p-toluenesulphonic acid and the like are suitable for the formation of pharmaceutically acceptable salts of basic compounds of formula I. The compounds of formula I and their pharmaceutically acceptable salts are metabotropic glutamate receptor antagonists and can be used for the treatment or prevention of acute and/or chronic neurological disorders, such as psychosis, schizophrenia, Alzheimer's disease, cognitive disorders and memory deficits. Other treatable indications are restricted brain function caused by bypass operations or transplants, poor blood supply to the brain, spinal cord injuries, head injuries, hypoxia caused by pregnancy, cardiac arrest and hypoglycaemia. Further treatable indications are acute and chronic pain, Huntington's chorea, ALS, dementia caused by AIDS, eye injuries, retinopathy, idiopathic parkinsonism or parkinsonism caused by medicaments as well as conditions which lead to glutamate-deficient functions, such as e.g. muscle spasms, convulsions, migraine, urinary incontinence, nicotine addiction, psychoses, opiate addiction, anxiety, vomiting, dyskinesia and depression.

The compounds of formula I and pharmaceutically acceptable salts thereof can be used as medicaments, e.g. in the form of pharmaceutical preparations. The pharmaceutical preparations can be administered orally, e.g. in the form of tablets, coated tablets, dragées, hard and soft gelatine capsules, solutions, emulsions or suspensions. However, the administration can also be effected rectally, e.g. in the form of suppositories, or parenterally, e.g. in the form of injection solutions.

The compounds of formula I and pharmaceutically acceptable salts thereof can be processed with pharmaceutically inert, inorganic or organic carriers for the production of pharmaceutical preparations. Lactose, corn starch or derivatives thereof, talc, stearic acid or its salts and the like can be used, for example, as such carriers for tablets, coated tablets, dragées and hard gelatine capsules. Suitable carriers for soft gelatine capsules are, for example, vegetable oils, waxes, fats, semi-solid and liquid polyols and the like; depending on the nature of the active substance no carriers are, however, usually required in the case of soft gelatine capsules. Suitable carriers for the production of solutions and syrups are, for example, water, polyols, sucrose, invert sugar, glucose and the like. Adjuvants, such as alcohols, polyols, glycerol, vegetable oils and the like, can be used for aqueous injection solutions of water-soluble salts of compounds of formula I, but as a rule are not necessary. Suitable carriers for suppositories are, for example, natural or hardened oils, waxes, fats, semi-liquid or liquid polyols and the like.

In addition, the pharmaceutical preparations can contain preservatives, solubilizers, stabilizers, wetting agents, emulsifiers, sweeteners, colorants, flavorants, salts for varying the osmotic pressure, buffers, masking agents or antioxidants. They can also contain still other therapeutically valuable substances.

As mentioned earlier, medicaments containing a compound of formula I or a pharmaceutically acceptable salt thereof and a therapeutically inert excipient are also an object of the present invention, as is a process for the production of such medicaments which comprises bringing one or more compounds of formula I or pharmaceutically acceptable salts thereof and, if desired, one or more other therapeutically valuable substances into a galenical dosage form together with one or more therapeutically inert carriers.

The dosage can vary within wide limits and will, of course, be fitted to the individual requirements in each particular case. In general, the effective dosage for oral or parenteral administration is between 0.01–20 mg/kg/day, with a dosage of 0.1–10 mg/kg/day being preferred for all of the indications described. The daily dosage for an adult human being weighing 70 kg accordingly lies between 0.7–1400 mg per day, preferably between 7 and 700 mg per day.

The present invention relates also to the use of compounds of formula I and of pharmaceutically acceptable salts thereof for the production of medicaments, especially for the control or prevention of acute and/or chronic neurological disorders of the aforementioned kind.

The compounds of the present invention are group II mGlu receptor antagonists. The compounds show activities, as measured in the assay described below, of 50 μM or less, typically 3 μM or less, and ideally of 0.5 μM or less. In the table below are described some specific pKi values of preferred compounds.

Compound	K_i mGlu2 (μM)
3-(8-Chloro-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-benzonitrile	0.028
3-[8-(4-Methyl-piperazin-1-yl)-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile	0.305
3-(8-Chloro-4-oxo-7-phenyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-benzonitrile	0.120
[4-(3-Cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-ylsulfanyl]-acetic acid methyl ester	0.051
2-[4-(3-Cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yl]-acetamide	0.037

-continued

Compound	K_i mGlu2 (μM)
3-(8-Methoxy-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-benzonitrile	0.046
3-(8-Cyanomethyl-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-benzonitrile	0.016
4-(3-Iodo-phenyl)-7-(2-methoxy-ethoxy)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one	0.021
4-(3-Imidazol-1-yl-phenyl)-7-(2-methoxy-ethoxy)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one	0.012
[RS]-3-[4-Oxo-8-(2-oxo-[1,3]dioxolan-4-ylmethoxy)-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile	0.035
7-Hydroxymethyl-4-(3-imidazol-1-yl-phenyl)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one	0.018
[4-(3-Imidazol-1-yl-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yloxy]-acetonitrile	0.009
8-(4-Fluoro-phenylethynyl)-7-hydroxymethyl-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one	0.009
7-(2-Hydroxy-ethoxy)-4-(3-imidazol-1-yl-phenyl)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one	0.032
8-(4-Fluoro-phenyl)-7-[4-(2-hydroxy-ethoxy)-piperidin-1-yl]-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one	0.100
8-(4-Fluoro-phenyl)-7-hydroxy-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one	0.007
8-(2-Fluoro-phenyl)-7-methoxy-4-[3-(2-methyl-imidazol-1-yl)-phenyl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one	0.138
5-[7-(2-Fluoro-phenyl)-8-methoxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-thiophene-2-carbonitrile	0.168
4-[7-(2-Fluoro-phenyl)-8-methoxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-pyridine-2-carbonitrile	0.033
8-(2-Fluoro-phenyl)-7-hydroxy-4-(3-[1,2,3]triazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one	0.028
8-(2-Fluoro-phenyl)-7-hydroxy-4-[3-(2-methyl-imidazol-1-yl)-phenyl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one	0.108
8-(2-Fluoro-phenyl)-7-hydroxy-4-[3-(2-methylsulfanyl-imidazol-1-yl)-phenyl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one	0.021
8-(2,5-Difluoro-phenyl)-7-methoxy-4-(3-[1,2,3]triazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one	0.012
8-(2-Fluoro-phenyl)-7-hydroxy-4-[3-(3-methyl-isoxazol-5-yl)-phenyl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one	0.015
3-[7-(2,5-Difluoro-phenyl)-8-hydroxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile	0.006
8-(4-Fluoro-phenylethynyl)-7-hydroxy-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one	0.013
8-(4-Fluoro-phenylethynyl)-7-hydroxy-4-(3-[1,2,3]triazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one	0.010

[^{3}H]-LY354740 Binding on mGlu2 Transfected CHO Cell Membranes

Transfection and Cell Culture

cDNA encoding the rat mGlu2 receptor protein in pBluescript II was obtained from Prof. S. Nakanishi (Kyoto, Japan), and subcloned into the eukaryotic expression vector pcDNA I-amp from Invitrogen (NV Leek, The Netherlands). This vector construct (pcD1mGR2) was co-transfected with a psvNeo plasmid encoding the gene for neomycin resistance, into CHO cells by a modified calcium phosphate method described by Chen & Okayama (1988). The cells were maintained in Dulbecco's Modified Eagle medium with reduced L-glutamine (2 mM final concentration) and 10% dialysed foetal calf serum from Gibco BRL (Basel, Switzerland). Selection was made in the presence of G-418 (1000 ug/ml final). Clones were identified by reverse transcription of 5 μg total RNA, followed by PCR using mGlu2 receptor specific primers 5'-atcactgcttgggtttctggcactg-3' and 5'-agcatcactgtgggtggcataggagc-3' in 60 mM Tris HCl (pH 10), 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 25 units/ml Taq Polymerase with 30 cycles annealing at 60° C. for 1 min., extention at 72° C. for 30 s, and 1min. 95° C.

Membrane Preparation

Cells, cultured as above, were harvested and washed three times with cold PBS and frozen at -80° C. The pellet was resuspended in cold 20 mM HEPES-NaOH buffer containing 10 mM EDTA (pH 7.4), and homogenised with a polytron (Kinematica, AG, Littau, Switzerland) for 10 s at 10 000 rpm. After centrifugation for 30 min. at 4° C., the pellet was washed once with the same buffer, and once with cold 20 mM HEPES-NaOH buffer containing 0.1 mM EDTA, (pH 7.4). Protein content was measured using the Pierce method (Socochim, Lausanne, Switzerland) using bovine serum albumin as standard.

[^{3}H]-LY354740 Binding

After thawing, the membranes were resuspended in cold 50 mM Tris-HCl buffer containing 2 mM $MgCl_2$ and 2 mM $CaCl_2$, (pH 7) (binding buffer). The final concentration of the membranes in the assays was 25 μg protein/ml. Inhibition experiments were performed with membranes incubated with 10 nM [^{3}H]-LY354740 at room temperature, for 1 hour, in presence of various concentrations of the compound to be tested. Following the incubations, membranes were filtered onto Whatmann GF/C glass fiber filters and washed 5 times with cold binding buffer. Non specific binding was measured in the presence of 10 μM DCG IV. After transfer of the filters into plastic vials containing 10 ml of Ultima-gold scintillation fluid (Packard, Zürich, Switzerland), the radioactivity was measured by liquid scintillation in a Tri-Carb 2500 TR counter (Packard, Zürich, Switzerland).

Data analysis

The inhibition curves were fitted with a four parameter logistic equation giving IC50 values, and Hill coefficients.

EXAMPLES

The following examples relate to the preparation of the (4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl esters (Synthetic Scheme C):

General Procedure A

Preparation of 4-iodo-2-nitroanilines by Iodination of 2-nitroanilines [According to Wilson, J. Gerald; Hunt, Frederick C. *Aust. J. Chem.* 1983, 36, 2317–25]

To a stirred solution of the 2-nitroaniline (1.0 mol) in HOAc (500 mL) containing anhydrous NaOAc (93–103 g, 1.125–1.25 mol), iodine monochloride (59–66 mL, 1.125–1.25 mol) in HOAc (300 mL) was added over 60 min. The reaction mixture was heated to the given temperature until thin layer chromatography (tlc) indicated complete conversion of the starting material, stirred for another 30 min at 23° C., then diluted slowly with H_2O (1000 mL) which caused the separation of the crystalline product. Stirring was continued for 1 h and the product was filtered off, washed free of HOAc and dried in vacuum at 60° C.

Example A1

5-Chloro-4-iodo-2-nitro-phenylamine

Prepared from 5-chloro-2-nitroaniline by iodination with iodine monochloride in HOAc/NaOAc according to the general procedure A (80° C.). Obtained as an orange solid.

MS (EI) 298 (M^+) and 300 [$(M+2)^+$]; mp 202–203° C. (dec.).

Example A2

4-Iodo-5-methyl-2-nitro-phenylamine

Prepared from 5-methyl-2-nitroaniline by iodination with iodine monochloride in HOAc/NaOAc according to the general procedure A (80° C.). Obtained as a red solid.

MS (EI) 278 (M^+); mp 154° C. (dec.).

Example A3

5-Amino-2-iodo-4-nitrobenzoic acid methyl ester

Prepared from 3-amino-4-nitrobenzoic acid methyl ester (22.25 g, {CAS-No. [99512-09-1]; prepared in two steps as follows: 3-hydroxy-4-nitrobenzoic acid (30 g, 164 mmol), NH_4Cl (21.91 g, 410 mmol) in 25% aq. NH_3 (180 mL) was heated in a steel autoclave at 160° C. for 7 h (internal pressure: 23 bar). Cooled to 23° C. and evaporated to dryness. Taken up in H_2O (200 mL), adjusted pH with conc. H_2SO_4 to pH 1, saturated with NaCl and extracted with EtOAc (6x750 mL), dried combined organic layer over $MgSO_4$. Filtration and removal of the solvent in vacuum left the sufficiently pure 3-amino-4-nitrobenzoic acid (22.26 g, 75%) as an orange solid. This material was suspended in MeOH (500 mL), conc. H_2SO_4 (3 mL) was added and the mixture was heated to 65° C. for 2.5 days. The solvent was removed in vacuum, the solid residue taken up in EtOAc, washed with sat. $NaHCO_3$-sol. and brine, followed by drying over $MgSO_4$. Removal of the solvent left the sufficiently pure 3-amino-4-nitrobenzoic acid methyl ester (22.25 g, 93%) as an orange solid.} by iodination with iodine monochloride in HOAc/NaOAc according to the general procedure A (35° C.). Obtained as an orange solid (29.38 g, 80%).

MS (EI) 322 (M^+); mp 168° C. (dec.).

General Procedure B

Preparation of (2-nitro-phenyl)-carbamic acid tert.-butyl esters from 2-nitroanilines

Method a: To a solution of diphosgene (4.1 mL, 34.1 mmol) in EtOAc (40 mL) at 0° C. was added a solution of the 4-iodo-2-nitroaniline (45.5 mmol) in EtOAc (200–500 mL), and the mixture was heated to reflux for 18 h. The solvent was removed in vacuum to leave a brown solid, which was triturated with hot hexane (200 mL). The solid material was filtered off and the filtrate was concentrated under reduced pressure to leave the pure 4-iodo-2-nitrophenylisocyanate as a yellow solid. This material was refluxed in a mixture of excess tert.-BuOH in CH_2Cl_2 for 2.5 h. Removal of the solvent left an orange solid which was purified by silica gel column chromatography with hexane/EtOAc to give the (4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester as a yellow solid.

Method b: To a mixture of the 4-iodo-2-nitroaniline (142 mmol) and cesium carbonate (55.5 g, 170 mmol) in 2-butanone (740 mL) was dropwise added a solution of Boc_2O (37.8 g, 173 mmol) in 2-butanone (170 mL) and the resulting mixture was stirred at 52° C. for 26 h. The solvent was removed in vacuum, the residue was treated with a mixture of H_2O (240 mL) and MeOH (240 mL) and extracted with hexane (3x500 mL). The combined hexane layer was washed with brine (200 mL) and all aqueous layers were reextracted with hexane (300 mL). All combined hexane layers were dried over $MgSO_4$, filtered and the solvent was removed in vacuum to give an orange solid, which was purified by silica gel column chromatography with hexane/EtOAc to give the (4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester as a yellow solid.

Method c: To a solution of the 4-iodo-2-nitroaniline (550 mmol) and DMAP (1.22 g, 10 mmol) in THF (1000 mL) at 23° C. was dropwise added within 70 min a solution of Boc_2O (246 g, 1128 mmol) in THF (500 mL) and stirring was continued at 23° C. for 75 min. The entire mixture was evaporated to dryness and dried at HV to leave a dark brown solid (253.59 g). This material was dissolved in DCM (1100 mL), cooled to 0° C. and TFA (84 mL, 1100 mmol) was added dropwise. The mixture was stirred at 0° C. for 2 h, poured into icecold sat. NaHCO3-sol., extracted with DCM, washed with brine and dried over MgSO4. Removal of the solvent in vacuum left a dark brown solid (199.71 g) which was coated on silica gel and purified by silica gel column chromatography with hexane/EtOAc to give the (4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester as a yellow solid.

Example B1

(5-Chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester

Prepared the isocyanate from 5-chloro-4-iodo-2-nitro-phenylamine (Example A1) (7.0 g, 23.45 mmol) with diphosgene (2.12 mL, 17.6 mmol) in EtOAc (30 mL), followed by treatment with tert.-BuOH (100 mL) in CH_2Cl_2 (100 mL) according to the general procedure B (method a). Obtained as a yellow solid (7.1 g, 76%).

MS (EI) 398 (M^+) and 400 [$(M+2)^+$]; mp 82–84° C.

Example B2

(4-Iodo-5-methyl-2-nitro-phenyl)-carbamic acid tert.-butyl ester

Prepared the isocyanate from 4-iodo-5-methyl-2-nitro-phenylamine (Example A2) (13.51 g, 48.6 mmol) with diphosgene (4.4 mL, 36.4 mmol) in EtOAc (50 mL), followed by treatment with tert.-BuOH (150 mL) and CH_2Cl_2 (150 mL) according to the general procedure B (method a). Obtained as a yellow solid (14.1 g, 77%).

MS (EI) 378 (M^+); mp 99–100° C.

Example B3

5-tert.-Butoxycarbonylamino-2-iodo-4-nitro-benzoic acid methyl ester

Prepared the isocyanate from 5-amino-2-iodo-4-nitro-benzoic acid methyl ester (Example A3) (5.5 g, 17 mmol) with diphosgene (1.55 mL, 13 mmol) in EtOAc (135 mL), followed by treatment with tert.-BuOH (20 mL) and CH_2Cl_2 (70 mL) according to the general procedure B (method a). Obtained as a yellow solid (5.2 g, 72%).

MS (ISP) 440 [$(M+NH_4)^+$]; mp 126° C.

Example B4

(5-Allyloxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester

Prepared the isocyanate from 5-allyloxy-4-iodo-2-nitro-phenylamine (Example E1) (9.0 g, 28.2 mmol) with diphosgene (2.6 mL, 21.2 mmol) in EtOAc (150 mL), followed by treatment with tert.-BuOH (80 mL) and CH_2Cl_2 (80 mL)

according to the general procedure B (method a). Obtained as a yellow solid (9.16 g, 77%).

MS (ISP) 421 [(M+H)$^+$] and 438 [(M+NH$_4$)$^+$]; mp 93–95° C.

Example B5

(4-Iodo-5-methoxy-2-nitro-phenyl)-carbamic acid tert.-butyl ester

Prepared the isocyanate from 4-iodo-5-methoxy-2-nitro-phenylamine (Example E2) (2.85 g, 9.69 mmol) with diphosgene (0.88 mL, 7.27 mmol) in EtOAc (52 mL), followed by treatment with tert.-BuOH (25 mL) and CH_2Cl_2 (25 mL) according to the general procedure B (method a). Obtained as a yellow solid (3.0 g, 79%).

MS (EI) 394 (M$^+$); mp 171° C.

Example B6

[4-Iodo-5-(2-methoxy-ethoxy)-2-nitro-phenyl]-carbamic acid tert.-butyl ester

Prepared the isocyanate from 4-iodo-5-(2-methoxy-ethoxy)-2-nitro-phenylamine (Example E3) (2.73 g, 8.08 mmol) with diphosgene (0.8 mL, 6.06 mmol) in EtOAc (50 mL), followed by treatment with tert.-BuOH (25 mL) and CH_2Cl_2 (25 mL) according to the general procedure B (method a). Obtained as a yellow solid (3.0 g, 86%).

MS (ISP) 439 [(M+H)$^+$], 456 [(M+NH$_4$)$^+$] and 461 [(M+Na)$^+$]; mp 109–111° C.

Example B7

[4-Iodo-5-(2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-2-nitro-phenyl]-carbamic acid tert.-butyl ester

Prepared the isocyanate from 4-Iodo-5-(2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-2-nitro-phenylamine (Example E4) (8.0 g, 17 mmol) with diphosgene (1.54 mL, 13 mmol) in EtOAc (100 mL), followed by treatment with tert.-BuOH (25 mL) and CH_2Cl_2 (25 mL) according to the general procedure B (method a). Obtained as a yellow oil (8.6 g, 89%).

MS (ISP) 588 [(M+NH$_4$)$^+$].

Example B8

(RS)-[5-(2,2-Dimethyl-[1,3]dioxolan-4-ylmethoxy)-2-nitro-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from (RS)-5-(2,2-dimethyl-[1,3]dioxolan-4-ylmethoxy)-2-nitro-4-phenylethynyl-phenylamine (Example K14) (2.678 g, 5.7 mmol), Cs_2CO_3 (2.23 g, 6.8 mmol) and Boc_2O (1.52 g, 7.0 mmol) in 2-butanone (36.5 mL) at 52° C. according to the general procedure B (method b). Obtained as a yellow foam (2.0 g, 75%).

MS (ISP) 469 [(M+H)$^+$] and 486 [(M+NH$_4$)$^+$]; mp 32° C.

Example B9

[Ve (R=CN; R"=Boc)]

(5-Cyanomethyl-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester

Prepared the isocyanate from (5-amino-2-iodo-4-nitro-phenyl)-acetonitrile (Example Vf (R=CN; R"=H)) (5.15 g, 17 mmol) with diphosgene (2.05 mL, 17 mmol) in EtOAc (150 mL), followed by treatment with tert.-BuOH (25 mL) and CH_2Cl_2 (25 mL) according to the general procedure B (method a). Obtained as a yellow solid (4.0 g, 58%).

MS (ISN) 402 [(M–H)$^-$]; mp 124–126° C.

Example B10

[5-(2-tert.-Butoxy-ethoxy)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester

Prepared the di-Boc-compound from 5-(2-tert.-butoxy-ethoxy)-4-iodo-2-nitro-phenylamine (Example E6) (13.9 g, 36.6 mmol) and Boc_2 O (16.35 g, 75 mmol), followed by treatment with 2 eq. TFA in CH_2Cl_2 according to the general procedure B (method c). Obtained as a yellow solid (14.96 g, 85%).

MS (ISP) 481 [(M+H)$^+$]; mp 113–116° C.

Example B11

[4-Iodo-5-(4-methoxy-benzyloxy)-2-nitro-phenyl]-carbamic acid tert.-butyl ester

Prepared the di-Boc-compound from 4-iodo-5-(4-methoxy-benzyloxy)-2-nitro-phenylamine (Example E7) (2.88 g, 7.20 mmol) and Boc_2O (3.30 g, 15.12 mmol), followed by treatment with 2 eq. TFA in CH_2Cl_2 according to the general procedure B (method c). Obtained as a waxy yellow solid (1.74 g).

MS (ISN) 499 [(M–H)$^-$].

The following procedures relate to the preparation of those (4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl esters bearing nitrogen substituents in the 5-position (Scheme D).

General Procedure C

Preparation of 5-N-substituted-(4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl esters

Method a: from (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester

(5-Chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) was stirred with the desired amine optionally with toluene or DMSO and/or DIPEA at temperature from 23° C. to 100–130° C. until tlc indicated complete disappearance of the chloride. The reaction was cooled to 23° C. poured into ice-water, the precipitate was filtered off, washed with water and dried in vacuum. In cases were the product did not precipitate, the mixture was extracted with EtOAc, washed with water and brine, dried over Na_2SO4. Filtration and removal of the solvent in vacuum left a crude product, which was purified by silica gel column chromatography with hexane/EtOAc to give the pure title compound.

Method b: from 5-chloro-4-iodo-2-nitro-phenylamine

A mixture of 5-chloro-4-iodo-2-nitro-phenylamine (Example A1) (1.49 g, 5.0 mmol), the desired amine (6–25 mmol) and an appropriate base, like for example $NaHCO_3$, K_2CO_3, Et_3N or DIPEA (10–15 mmol) was stirred in DMSO, DMF or toluene (20-50 mL) at 60–130° C. until tlc indicated complete disappearance of the chloride. The reaction was cooled to 23° C. poured into ice-water, neutralized with 1N HCl, the precipitate was filtered off, washed with water and dried in vacuum. In cases were the product did not

precipitate, the mixture was extracted with EtOAc, washed with water and brine, dried over Na_2SO4. Filtration and removal of the solvent in vacuum left a crude product, which was purified by silica gel column chromatography with hexane/EtOAc to give the pure title compound. Protection of the amino-group was achieved by following the general procedure B.

Example C1

[4-Iodo-5-(4-methyl-piperazin-1-yl)-2-nitro-phenyl]-carbamic acid tert.-butyl ester

Prepared from (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) (2.39 g, 6.0 mmol) and 1-methylpiperazine (1.60 mL, 15 mmol) in toluene (4.5 mL) at 110° C. for 18 h according to the general procedure C (method a). Obtained as a yellow solid (2.2 g).

MS (ISP) 463 [(M+H)$^+$]; mp 134–136° C.

Example C2

(4-Iodo-2-nitro-5-thiomorpholin-4-yl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) (2.0 g, 5.0 mmol) and thiomorpholine (2.6 mL) in toluene (3.8 mL) DIPEA (1.7 mL) at 115° C. for 48 h according to the general procedure C (method a). Obtained as a yellow solid (1.1 g).

MS (ISP) 466 [(M+H)$^+$]; mp 132–134° C.

Example C3

(4-Iodo-5-morpholin-4-yl-2-nitro-phenyl)-carbamic acid tert.-butyl ester

Prepared from (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) (2.0 g, 5.0 mmol) and morpholine (10 mL) at reflux for 3 h according to the general procedure C (method a). Obtained as a yellow solid (0.805 g).

MS (ISP) 450 [(M+H)$^+$]; mp 43–44° C.

Example C4

[5-(1,4-Dioxa-8-aza-spiro [4.5]dec-8-yl)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester

Prepared from (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) (3.0 g, 7.53 mmol), 1,4-dioxa-8-aza-spiro(4,5)decane (4.82 mL, 37.63 mmol) and DIPEA (2.58 mL, 15.0 mmol) in toluene (4 mL) at reflux for 6 h according to the general procedure C (method a). Obtained as an orange solid (4.0 g).

MS (ISP) 506 [(M+H)$^+$]; mp 132–134° C.

Example C5

[4-Iodo-5-(4-methoxy-piperidin-1-yl)-2-nitro-phenyl]-carbamic acid tert.-butyl ester

Prepared from 5-chloro-4-iodo-2-nitro-phenylamine (Example A1) (6.91 g, 23.15 mmol), 4-methoxypiperidine (4.0 g, 34.73 mmol) and $NaHCO_3$ (5.83 g, 69.45 mmol) in DMSO (230 mL) at 100° C. according to the general procedure C (method b). The obtained brown solid (7.95 g) was converted to the title compound according to the general procedure B (method c). Obtained as a yellow solid (6.55 g).

MS (ISP) 478 [(M+H)$^+$]; mp 133–135° C.

Example C6

{5-[(2-Hydroxy-ethyl)-methyl-amino]-4-iodo-2-nitro-phenyl}-carbamic acid tert.-butyl ester

Prepared from (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) (1.99 g, 5.0 mmol) and 2-methylaminoethanol (2.00 mL, 25.0 mmol) in DMSO (2.5 mL) at 23° C. according to the general procedure C (method a). Obtained as a yellow gum (1.88 g).

MS (ISP) 438 [(M+H)$^+$].

Example C7

[5-(4-Hydroxy-piperidin-1-yl)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester

Prepared from (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) (1.99 g, 5 mmol) and 4-hydroxypiperidine (2.53 g, 25 mmol) in DMSO (2.5 mL) at 23° C. according to the general procedure C (method a). Obtained as a yellow solid (1.88 g).

MS (EI) 463 (M$^+$); mp 58–60° C.

Example C8

{5-[4-(2-Hydroxy-ethoxy)-piperidin-1-yl]-4-iodo-2-nitro-phenyl}-carbamic acid tert.-butyl ester

Prepared from (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) (2.2 g, 5.5 mmol), 4-(2-hydroxyethoxy)piperidine {CAS-No. [40256-14-2]} (800 mg, 5.5 mmol) and Et_3N (2.3 mL, 16.5 mmol) in DMSO (2.3 mL) at 23° C. according to the general procedure C (method a). Obtained as a yellow solid (1.65 g).

MS (EI) 507 (M$^+$); mp 64–65° C.

Example C9

[5-(cis-3,4-Dihydroxy-pyrrolidin-1-yl)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester

Prepared in two steps as follows: (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) (8.73 g, 21.9 mmol) was reacted with 3-pyrroline (2.0 mL, 26.3 mmol, 70% pure, contains 30% pyrrolidine), Et_3N (9.12 mL, 65.7 mmol) in DMSO (14 mL) and EtOH (5 mL) at 23° C. according to the general procedure C (method a) to give a 7:3 mixture of [5-(2,5-dihydro-pyrrol-1-yl)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester and (4-iodo-2-nitro-5-pyrrolidin-1-yl-phenyl)-carbamic acid tert.-butyl ester Obtained as a yellow solid (8.57 g). Part (4.31 g) of this material was dihydroxylated by reaction with NMO (1.28 g, 11.0 mmol), 2.5% OsO_4 in t-BuOH (1 mL, 0.1 mmol) and K_2OsO_4 (40 mg, 0.1 mmol) in acetone (250 mL) and H_2O (100 mL) at 23° C. for 6 days. Obtained the title compound as an amorphous yellow substance (2.50 g).

MS (ISP) 466 [(M+H)$^+$].

Example C10

[5-(2-Hydroxy-ethylamino)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester

Prepared from (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) (3.99 g, 10 mmol) and ethanolamine (3.01 mL, 50 mmol) in DMSO (20 mL) at 23° C. according to the general procedure C (method a). Obtained as a yellow solid (4.53 g).

MS (ISP) 424 [(M+H)$^+$]; mp 130–148° C.

Example C11

[5-((R)-3-Hydroxy-pyrrolidin-1-yl)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester

Prepared from (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1), (R)-3-hydroxypyrrolidine hydrochloride and Et_3N in DMSO at 23° C. according to the general procedure C (method a). Obtained as a yellow solid (3.153 g).

MS (ISP) 450 [$(M+H)^+$]; mp 158° C. (dec.).

The following procedures relate to the preparation of those (4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl esters bearing sulfur substituents in the 5-position (Synthetic Scheme D):

General Procedure D

Preparation of 5-S-substituted-(4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl esters from (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester

To a solution of the thiol (2.2 mmol) in DMF was added NaOMe-sol. (5.4M in MeOH, 0.41 mL, 2.2 mmol) followed by (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) (797 mg, 2.0 mmol) and stirring was continued at 23° C. until tlc indicated complete disappearance of the chloride. Poured into ice-cold 5% citric acid, extracted with EtOAc, washed with sat. $NaHCO_3$-sol., brine, dried over $MgSO_4$. Removal of the solvent left an orange oil, which was purified by silica gel column chromatography with hexane/EtOAc to give the pure title compound.

Example D1

[5-(2-Dimethylamino-ethylsulfanyl)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester

Prepared from (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) (399 mg, 1.0 mmol), 2-dimethylaminoethanolethiol hydrochloride (312 mg, 2.2 mmol) and NaOMe solution 5.4M in MeOH (0.8 mL, 8.8 mmol) in DMF (2 mL) according to the general procedure D. Obtained as a yellow solid (306 mg).

MS (ISP) 468 [$(M+H)^+$]; mp 144° C.

Example D2

(5-tert.-Butoxycarbonylamino-2-iodo-4-nitro-phenylsulfanyl)-acetic acid methyl ester

Prepared from (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) (797 mg, 2.0 mmol), methyl thioglycolate (0.2 mL, 2.2 mmol) and NaOMe solution 5.4M in MeOH (0.41 mL, 2.2 mmol) in DMF (2 mL) according to the general procedure D. Obtained as a yellow solid (847 mg).

MS (EI) 468 (M^+); mp 110–112° C.

The following procedures relate to the preparation of those (4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl esters bearing oxygen substituents in the 5-position (Scheme E)

General Procedure E:

Preparation of 5-O-substituted-4-iodo-2-nitro-phenylamines from 5-chloro-4-iodo-2-nitro-phenylamine

To a suspension of KOH (85%, 3.62–7.96 g, 55–121 mmol) in DMSO (50 mL) was added the alcohol (125–500 mmol) and the mixture was stirred at 23° C. until all KOH had dissolved. 5-Chloro-4-iodo-2-nitro-phenylamine (Example A1) (15.0 g, 50 mmol) was added in small portions and the resulting dark red clear solution was stirred at 23–60° C. until tlc indicated complete disappearance of the chloride. Poured into ice-cold 1N HCl or ice-cold sat. NH_4Cl-sol., extracted with EtOAc or $CHCl_3$, washed with 1N HCl or sat. NH_4Cl-sol. and brine, dried over $MgSO_4$. Removal of the solvent left a dark red solid, which was purified by silica gel column chromatography to give the pure title compound.

Example E1

5-Allyloxy-4-iodo-2-nitro-phenylamine

Prepared from 5-chloro-4-iodo-2-nitro-phenylamine (Example Al) (15.0 g, 50 mmol), allyl alcohol (50 mL) and KOH (7.96 g, 121 mmol) in DMSO (50 mL) according to the general procedure E. Obtained as an orange solid (9.38 g).

MS (EI) 320 (M^+); mp 74° C.

Example E2

4-Iodo-5-methoxy-2-nitro-phenylamine

Prepared from 5-chloro-4-iodo-2-nitro-phenylamine (Example A1) (2.98 g, 10 mmol), methanol (10 mL) and KOH (1.45 g, 22 mmol) in DMSO (10 mL) according to the general procedure E. Obtained as an orange solid (2.9 g).

MS (ISP) 295 [$(M+H)^+$] and 312 [$(M+NH_4)^+$]; mp 189° C.

Example E3

4-Iodo-5-(2-methoxy-ethoxy)-2-nitro-phenylamine

Prepared from 5-chloro-4-iodo-2-nitro-phenylamine (Example A1) (2.98 g, 10 mmol), 2-methoxyethanol (7.9 mL, 100 mmol) and KOH (1.45 g, 22 mmol) in DMSO (8 mL) according to the general procedure E. Obtained as an orange solid (2.8 g).

MS (ISN) 337 [$(M-H)^-$]; mp 121–122° C.

Example E4

4-Iodo-5-(2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-2-nitro-phenylamine

Prepared from 5-chloro-4-iodo-2-nitro-phenylamine (Example A1) (9.48 g, 32 mmol), tetraethyleneglycol monomethyl ether (19 g, 91 mmol) and KOH (2.31 g, 35 mmol) in DMSO (25 mL) at 60° C. according to the general procedure E. Obtained as a red oil (8.4 g).

MS (ISP) 471 [$(M+H)^+$].

Example E5

(RS)-5-(2,2-Dimethyl-[1,3]dioxolan-4-ylmethoxy)-4-iodo-2-nitro-phenylamine

Prepared from 5-chloro-4-iodo-2-nitro-phenylamine (Example A1) (4.48 g, 15 mmol), D,L-α, β-isopropylidene-glycerol (10 mL, 81 mmol) and KOH (1.01 g, 18 mmol) in DMSO (10 mL) at 23° C. according to the general procedure E. Obtained as a yellow solid (4.9 g).

MS (ISN) 393 [$(M-H)^-$]; mp 151° C.

Example E6

5-(2-tert.-Butoxy-ethoxy)-4-iodo-2-nitro-phenylamine

Prepared from 5-chloro-4-iodo-2-nitro-phenylamine (Example A1) (14.9 g, 50 mmol), 2-tert.-butoxyethanol

(29.5 g, 250 mmol) and KOH (3.99 g, 60 mmol) in DMSO (25 mL) at 23° C. according to the general procedure E. Obtained as a yellow solid (14.3 g).

MS (ISP) 381 [(M+H)$^+$]; mp 144–146° C.

Example E7

4-Iodo-5-(4-methoxy-benzyloxy)-2-nitro-phenylamine

Prepared from 5-chloro-4-iodo-2-nitro-phenylamine (Example Al) (5.97 g, 20 mmol), 4-methoxybenzyl alcohol (4.98 mL, 40 mmol) and KOH (1.58 g, 24 mmol) in DMSO (30 mL) at 23° C. according to the general procedure E. Obtained as a yellow-brown solid (2.94 g).

MS (ISN) 399 [(M–H)$^-$]; mp 183° C.

The following examples relate to the preparation of (5-tert.-butoxycarbonylamino-2-iodo-4-nitro-phenyl)-acetic acid methyl ester and (5-cyanomethyl-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Synthetic Scheme F):

Example XVI

(R=CO_2Me: R'=Me: R"=Boc)

2-(5-tert.-Butoxycarbonylamino-2-iodo-4-nitro-phenyl)-malonic acid dimethyl ester

To a solution of KOBut (0.56 g, 5.02 mmol) in DMSO (3 mL) was added dimethyl malonate (0.58 mL, 5.02 mmol) followed by (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) (1.00 g, 2.51 mmol) and the resulting dark red clear solution was stirred at 100° C. until tlc indicated complete disappearance of the chloride. Poured into ice-cold 5% citric acid (100 mL), extracted with EtOAc (2×100 mL), washed with brine, dried over $MgSO_4$. Removal of the solvent left a yellow oil, which was purified by silica gel column chromatography with hexane/EtOAc 4:1 to give the pure title compound as a yellow gum (1.13 g, 91%)

MS (ISP) 512 [(M+NH_4)$^+$] and 517 [(M+Na)$^+$].

Example XVII

(R=CN; R'=Et; R"=H)

(RS)-(5-Amino-2-iodo-4-nitro-phenyl)-cyano-acetic acid ethyl ester

Prepared as described for example XVI from 5-chloro-4-iodo-2-nitro-phenylamine (Example A1) (14.9 g, 50 mmol), ethyl cyanoacetate (14.7 mL, 100 mmol) and KOBut (11.2 g, 100 mmol) in DMSO (60 mL) at 100° C. for 2 h. Obtained as a dark brown gum.

MS (EI) 375 (M$^+$).

Example Ve

(R=CO_2Me; R"=Boc)

(5-tert.-Butoxycarbonylamino-2-iodo-4-nitro-phenyl)-acetic acid methyl ester

A mixture of 2-(5-tert.-butoxycarbonylamino-2-iodo-4-nitro-phenyl)-malonic acid dimethyl ester (Example XVI (R=CO_2Me; R'=Me; R"=Boc)) (3.34 g, 6.76 mmol), LiCl (573 mg, 13.52 mmol) and H_2O (0.122 mL, 6.76 mmol) in DMSO (46 mL) was stirred at 100° C. for 7 h. Poured into ice-water, extracted twice with EtOAc, washed with brine, dried over $MgSO_4$.

Removal of the solvent left a yellow oil, which was purified by silica gel column chromatography with hexane/EtOAc 9:1 to give the pure title compound as a yellow solid (1.37 g, 47%).

MS (EI) 436 (M+); mp 93° C.

Example Vf

(R=CN; R"=H)

(5-Amino-2-iodo-4-nitro-phenyl)-acetonitrile

Prepared as described for example Ve from (RS)-(5-amino-2-iodo-4-nitro-phenyl)-cyano-acetic acid ethyl ester (Example XVII (R=CN; R'=Et; R"=H)) (20.62 g, 55 mmol) and LiCl (9.33 g, 220 mmol) in DMSO (370 mL) and H_2O (4.4 mL) at 120° C. for 2.5 h. Obtained as a green-brown solid.

MS (EI) 303 (M$^+$); mp 145–183° C.

The following examples relate to the preparation of (5-hydroxymethyl-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester, the corresponding THP-ether, as well as the (5-dimethylaminomethyl-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester via the intermediate chloride (Synthetic Scheme F):

Example Vh

(5-Hydroxymethyl-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester

$LiBH_4$ (0.32 g, 14.78 mmol) was added to a solution of 5-tert.-butoxycarbonylamino-2-iodo-4-nitro-benzoic acid methyl ester (Example B3) (3.12 g, 7.39 mmol) and MeOH (0.6 mL, 14.78 mmol) in THF (44 mL) at 0° C. The reaction mixture was stirred at 0° C. for 15 min. Poured into 5% citric acid, extracted twice with EtOAc, washed with sat. $NaHCO_3$-sol. and brine, dried over $MgSO_4$. Removal of the solvent left a yellow oil, which was purified by silica gel column chromatography with cyclohexane/EtOAc 4:1 to give the pure title compound as a yellow solid (2.64 g, 91%).

MS (ISP) 412 [(M+NH_4)$^+$]; mp>250° C.

Example Vi

(RS)-[4-Iodo-2-nitro-5-(tetrahydro-pyran-2-yloxymethyl)-phenyl]-carbamic acid tert.-butyl ester

To a mixture of (5-hydroxymethyl-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example Vh) (394 mg, 1.0 mmol) and 3,4-dihydro-2H-pyran (0.11 mL, 1.2 mmol) in DCM (5 mL) at 0° C. was added p-TsOH.H_2O (ca. 5 mg) and the reaction was stirred at 0° C. for 1 Diluted with EtOAc, washed with sat. $NaHCO_3$-sol. and brine, dried over $MgSO_4$. Removal of the solvent in vacuum left a yellow oil, which was purified by silica gel column chromatography with hexane/EtOAc 9:1 to give the pure title compound as a yellow gum (470 mg, 98%).

MS (ISN) 477 [(M–H)$^-$].

Example VI

(5-Dimethylaminomethyl-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester

To a mixture of (5-hydroxymethyl-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example Vh), LiCl (3 eq.) and pyridine (2 eq.) in DMF at 0° C. was added methanesulfonyl chloride (1.5 eq.) and the reaction was stirred at 23° C. for 24 h. Me_2NH in EtOH (10 eq.) was added and stirring was continued for 24 h. Diluted with EtOAc, washed with sat. $NaHCO_3$-sol. and brine, dried over $MgSO_4$. Removal of the solvent in vacuum left a yellow oil, which was purified by silica gel column chromatography with cyclohexane/EtOAc 3:1 to give the pure title compound as a yellow oil (421 mg).

MS (ISP) 422 [(M+H)$^+$].

The following examples relate to the preparation of (2-amino-4-aryl-phenyl)-carbamic acid tert.-butyl esters, [2-amino-4-(1-alkenyl)-phenyl]-carbamic acid tert.-butyl esters and (2-amino-4-aroyl-phenyl)-carbamic acid tert.-butyl esters in regioisomerically pure fashion (Synthetic Scheme B):

General Procedure F:

Preparation of (4-aryl-2-nitro-phenyl)-carbamic acid tert.-butyl esters by direct Suzuki-coupling of (4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl esters with arylboronic acids

A mixture of the (4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (3.0 mmol), the arylboronic acid (4.5 mmol) and $PdCl_2(PPh_3)_2$ (2 mol %) was refluxed in 1,4-dioxane (25 mL) and 2M Na_2CO_3-sol. (7.5 mL) [or alternatively with 1M $NaHCO_3$-sol. (7.5 mL), LiCl (6.0 mmol) and $(Ph_3P)_4Pd$ (3 mol %) in DME (30 mL); also possible with Et_3N (9.0 mmol), $Pd(OAc)_2$ (3 mol %), PPh_3 (6 mol %) in DMF (10 mL) at 100° C.] until tlc indicated complete conversion of the iodide. The mixture was transferred into a separating funnel, H_2O (25 mL) was added and the product was extracted with ether or EtOAc (3x30 mL). The combined organic layers were washed with brine (50 mL) and dried over Na_2SO_4. Removal of the solvent left a brown residue, which was purified by silica gel column chromatography with cyclohexane/ether or cyclohexane/EtOAc to give the title compound.

Example F1

(2-Chloro-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) (1.20 g, 3.00 mmol) and phenyl boronic acid (0.62 g, 3.30 mmol) according to the general procedure F. Obtained as a yellow oil (843 mg).

MS (EI) 348 (M$^+$) and 350 [(M+2)$^+$].

Example F2

(2-Methyl-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (4-iodo-5-methyl-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B2) (1.135 g, 3 mmol) and phenylboronic acid (630 mg, 3.3 mmol) according to the general procedure F. Obtained as a yellow oil (971 mg).

MS (EI) 328 (M$^+$).

Example F3

(RS)-{4'-Fluoro-5-nitro-2-[4-(tetrahydro-pyran-2-yloxy)-piperidin-1-yl]-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (RS)-{4-iodo-2-nitro-5-[4-(tetrahydro-pyran-2-yloxy)-piperidin-1-yl]-phenyl}-carbamic acid tert.-butyl ester [RO-69-4319/000, prepared from [5-(4-hydroxy-piperidin-1-yl)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester (Example C7) by treatment with 3,4-dihydro-2H-pyran and cat. TsOH.H_2O in DCM at 0° C.](1.09 g, 2.0 mmol) and 4-fluorophenylboronic acid according to the general procedure F. Obtained as an orange solid (894 mg).

MS (ISP) 516 [(M+H)$^+$]; mp 144–146° C.

Example F4

(RS)-(4'-Fluoro-5-nitro-2{4-[2-(tetrahydro-pyran-2-yloxy)-ethoxy]-piperidin-1-yl }-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (RS)-(4-iodo-2-nitro-5-{4-[2-(tetrahydro-pyran-2-yloxy)-ethoxy]-piperidin-1yl}-phenyl)-carbamic acid tert.-butyl ester [RO-69-4355/000, prepared from {5-[4-(2-hydroxy-ethoxy)-piperidin-1-yl]-4-iodo-2-nitro-phenyl}-carbamic acid tert.-butyl ester (Example C8) by treatment with 3,4-dihydro-2H-pyran and cat. TsOH.H_2O in DCM at 0° C.] (950 mg, 1.87 mmol) and 4-fluorophenylboronic acid (314 mg, 2.25 mmol) according to the general procedure F. Obtained as a viscous orange oil (930 mg).

MS (ISP) 560 [(M+H)$^+$]; mp 144–146° C.

Example F5

(RS)-[4'-Fluoro-5-nitro-2-(tetrahydro-pyran-2-yloxymethyl)-biphenyl-4-yl -carbamic acid tert.-butyl ester

Prepared from (RS)-[4-iodo-2-nitro-5-(tetrahydro-pyran-2-yloxymethyl)-phenyl]-carbamic acid tert.-butyl ester (Example Vi) and 4-fluorophenylboronic acid according to the general procedure F. Obtained as an orange oil (1.24 g).

Example F6

(2-Cyanomethoxy-4'-fluoro-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (5-cyanomethoxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example S2) (838 mg, 2.0 mmol) and 4-fluorophenylboronic acid (392 mg, 2.8 mmol) according to the general procedure F. Obtained as a yellow solid (333 mg).

MS (ISP) 405 [(M+NH_4)$^+$]; mp 148° C.

Example F7

(2-Dimethylaminomethyl-4'-fluoro-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (5-dimethylaminomethyl-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example VI) and 4-fluorophenylboronic acid according to the general procedure F. Obtained as a yellow solid (1.01 g).

Example F8

[2-(2,2-Dimethyl-tetrahydro-[1,3]dioxolo [4,5-c] pyrrol-5-yl)-4'-fluoro-5-nitro-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [5-(cis-2,2-dimethyl-tetrahydro-[1,3] dioxolo [4,5-c]pyrrol-5-yl)-4-iodo-2-nitro-phenyl]-carbamic acid tert-butyl ester [RO-69–4741/000, prepared from [5-(cis-3,4-dihydroxy-pyrrolidin-1-yl)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester (Example C9) by treatment with 2,2-dimethoxypropane and cat. TsOH.H_2O in DMF at 23° C.](845 mg, 1.67 mmol) and 4-fluorophenylboronic acid (327 mg, 2.34 mmol) according to the general procedure F. Obtained as a yellow solid (643 mg).

MS (ISP) 474 [(M+H)$^+$]; mp 119° C.

Example F9

(4'-Fluoro-2-methoxy-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (4-iodo-5-methoxy-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B5) (3.68 g, 9.34

mmol) and 4-fluorophenylboronic acid (3.61 g, 25.8 mmol) according to the general procedure F. Obtained as a yellow solid (2.69 g).

MS (ISN) 361 [(M–H)^{31}]; mp 250° C.

Example F10

[2-(1,4-Dioxa-8-aza-spiro[4.5]dec-8-yl)-4'-fluoro-5-nitro-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [5-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester (Example C4) (4.0 g, 7.02 mmol) and 4-fluorophenylboronic acid (1.33 g, 9.5 mmol) according to the general procedure F. Obtained as a yellow solid (2.43 g).

mp 213° C. (dec.).

Example F11

(4'-Fluoro-2-methyl-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (4-iodo-5-methyl-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B2) (756 mg, 2.0 mmol) and 4-fluorophenylboronic acid (420 mg, 3.0 mmol) according to the general procedure F. Obtained as an amorphous yellow substance (611 mg).

MS (ISN) 345 [(M–H)$^-$].

Example F12

(4-tert.-Butoxycarbonylamino-4'-fluoro-5-nitro-biphenyl-2-yloxy)-acetic acid tert.-butyl ester

Prepared from (5-tert.-butoxycarbonylamino-2-iodo-4-nitro-phenoxy)-acetic acid tert.-butyl ester (Example S1) (2.14 g, 4.33 mmol) and 4-fluorophenylboronic acid (728 mg, 5.2 mmol) according to the general procedure F. Obtained as an orange solid (1.80 g).

MS (ISN) 461 [(M–H)$^-$]; mp 92–93° C.

Example F13

(2-Chloro-4'-fluoro-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) and 4-fluorophenylboronic acid according to the general procedure F. Obtained as a yellow solid (625 mg).

MS (EI) 366 (M$^+$).

Example F14

[4'-Fluoro-2-(2-methoxy-ethoxy)-5-nitro-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [4-iodo-5-(2-methoxy-ethoxy)-2-nitro-phenyl-carbamic acid tert.-butyl ester (Example B6) and 4-fluorophenylboronic acid according to the general procedure F. Obtained as a yellow solid (1.833 g).

MS (EI) 406 (Me).

Example F15

[2-(2-tert.-Butoxy-ethoxy)-4'-fluoro-5-nitro-biphenyl-4-yl -carbamic acid tert.-butyl ester

Prepared from [5-(2-tert.-butoxy-ethoxy)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester (Example B10) and 4-fluorophenylboronic acid according to the general procedure F. Obtained as a yellow solid (735 mg).

MS (ISP) 449 [(M+H)$^+$].

Example F16

[4'-Fluoro-5-nitro-2-(2-oxo-oxazolidin-3-yl)-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [4-iodo-2-nitro-5-(2-oxo-oxazolidin-3-yl)-phenyl]-carbamic acid tert-butyl ester [RO-69-6758/000, prepared from [5-(2-hydroxy-ethylamino)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester (Example C10) by treatment with 1,1'-carbonyldiimidazole in dioxane and then in pyridine with cat. DMAP each at 100° C.](503 mg, 1.12 mmol) and 4-fluorophenylboronic acid (235 mg, 1.68 mmol) according to the general procedure F. Obtained as a yellow solid (310 mg).

MS (ISN) 416 [(M–H)$^-$]; mp 201° C.

Example F17

(4'-Fluoro-2-methoxy-2'-methyl-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (4-iodo-5-methoxy-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B5) and 4-fluoro-2-methyl-phenylboronic acid according to the general procedure F. Obtained as a yellow solid (699 mg).

MS (EI) 376 (M$^+$).

Example F18

(2-tert.-Butoxy-4'-fluoro-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (5-tert.-butoxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example S4) (1.4 g, 3.21 mmol) and 4-fluorophenylboronic acid (0.67 g, 4.42 mmol) according to the general procedure F. Obtained as an amorphous yellow substance (1.2 g).

MS (EI) 404 (M$^+$).

Example F19

(2-tert.-Butoxy-2'-fluoro-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (5-tert.-butoxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example S4) (1.4 g, 3.21 mmol) and 2-fluorophenylboronic acid (0.67 g, 4.83 mmol) according to the general procedure F. Obtained as an amorphous yellow substance (960 mg).

MS (EI) 404 (M$^+$).

Example F20

(RS)-{4'-Fluoro-5-nitro-2-[(R)-3-(tetrahydro-pyran-2-yloxy)-pyrrolidin-]-yl]-biphenyl-4yl}-carbamic acid tert.-butyl ester

Prepared from (RS)-{4-iodo-2-nitro-5-[(R)-3-(tetrahydro-pyran-2-yloxy)-pyrrolidin-1-yl]-phenyl}-carbamic acid tert-butyl ester [RO-69-6376/000, prepared from 15-((R)-3-hydroxy-pyrrolidin-1-yl)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester (Example C11) by treatment with 3,4-dihydro-2H-pyran and cat. TsOH.H_2O in DCM at 0° C.] and 4-fluorophenylboronic acid according to the general procedure F. Obtained as a yellow solid (1.053 g).

MS (ISP) 502 [(M+H)$^+$].

Example F21

(2'-Fluoro-2-methoxy-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (4-iodo-5-methoxy-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B5) (1.00 g, 2.54 mmol) and 2-fluorophenylboronic acid (0.60 g, 4.32 mmol) according to the general procedure F. Obtained as an amorphous yellow substance (687 mg).

MS (EI) 362 (M$^+$).

Example F22

[2-(1,4-Dioxa-8-aza-spiro[4.5]dec-8-yl)-2'-fluoro-5-nitro-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [5-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester (Example C4) (1.89 g, 3.47 mmol) and 2-fluorophenylboronic acid (0.63 g, 4.49 mmol) according to the general procedure F. Obtained as a yellow solid (1.46 g).

MS (ISP) 474 [(M+H)$^+$]; mp 164° C.

Example F23

(2',5'-Difluoro-2-methoxy-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (4-iodo-5-methoxy-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B5) (3.94 g, 10 mmol) and 2,5-difluorophenylboronic acid (2.21 g, 14 mmol) according to the general procedure F. Obtained as an amorphous yellow substance (1.05 g).

MS (ISN) 379 [(M−H)$^-$].

Example F24

[2'-Fluoro-2-(2-methoxy-ethoxy)-5-nitro-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [4-iodo-5-(2-methoxy-ethoxy)-2-nitro-phenyl]-carbamic acid tert.-butyl ester (Example B6) and 2-fluorophenylboronic acid according to the general procedure F. Obtained as a yellow solid (3.63 g).

MS (ISN) 405 [(M−H)$^-$].

Example F25

(RS)-[2'-Fluoro-5-nitro-2-(tetrahydro-pyran-2-yloxymethyl)-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from (RS)-[4-iodo-2-nitro-5-(tetrahydro-pyran-2-yloxymethyl)-phenyl]-carbamic acid tert.-butyl ester (Example Vi) and 2-fluorophenylboronic acid according to the general procedure F. Obtained as a yellow liquid (2.606 g).

MS (ISN) 445 [(M−H)$^-$].

Example F26

[2'-Fluoro-2-(4-methoxy-benzyloxy)-5-nitro-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [4-iodo-5-(4-methoxy-benzyloxy)-2-nitro-phenyl]-carbamic acid tert.-butyl ester (Example B11) (1.69 g, 3.38 mmol) and 2-fluorophenylboronic acid (0.61 g, 4.39 mmol) according to the general procedure F. Obtained as a yellow foam (940 mg).

MS (ISP) 469 [(M+H)$^+$].

Example F27

(2-tert.-Butoxy-2',5'-difluoro-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (5-tert.-butoxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example S4) (3.00 g, 6.88 mmol) and 2,5-difluorophenylboronic acid (2.23 g, 14.1 mmol) according to the general procedure F. Obtained as an amorphous yellow substance (2.30 g).

MS (ISN) 421 [(M−H)$^-$].

General Procedure G

Preparation of (4-aryl-2-nitro-phenyl)-carbamic acid tert.-butyl esters by Suzuki-coupling of (4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl esters with bis(pinacolato)diboron and subsequent reaction with aryl halides

A mixture of the (4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (2.0 mmol), bis(pinacolato)diboron (2.2 mmol), KOAc (6.0 mmol) and $PdCl_2(PPh_3)_2$ (3 mol %) in 1,4-dioxane (25 mL) was stirred at 100° C. until tlc indicated complete conversion of the iodide [cf. *Tetr. Lett.* 1997, 38, 3841–3844]. After addition of the aryl halide (4.0 mmol), $PdCl_2(PPh_3)_2$ (3 mol %) and 2M Na_2CO_3-sol. (7.5 mL) the mixture was stirred at 100° C. until tlc indicated complete conversion of the intermediate boronic ester. The mixture was transferred into a separating funnel, H_2O (30 mL) was added and the product was extracted with ether or EtOAc (3x50 mL). The combined organic layers were washed with brine (100 mL) and dried over Na_2SO_4. Removal of the solvent left a brown residue, which was purified by silica gel column chromatography with cyclohexane/ether or cyclohexane/EtOAc to give the title compound.

General Procedure H

Preparation of (4-aroyl-2-nitro-phenyl)-carbamic acid tert.-butyl esters by carbonylative Suzuki-coupling of (4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl esters with aryl boronic acids

A mixture of the (4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (1.0 mmol), aryl boronic acid (1.1 mmol), K_2CO_3 (3.0 mmol) and $PdCl_2(PPh_3)_2$ (3 mol %) in anisole (6 mL was stirred at 80° C. under a CO-atmosphere until thin layer chromatography indicated complete conversion of the iodide [cf. *Tetr. Lett.* 1993, 34, 7595–7598]. The mixture was transferred into a separating funnel, H_2O (30 mL) was added and the product was extracted with EtOAc (2x100 mL). The combined organic layers were washed with brine (50 mL) and dried over Na_2SO_4. Removal of the solvent left a yellow residue, which was purified by silica gel column chromatography with or hexane/EtOAc to give the title compound.

General Procedure I

Preparation of (4-aryl-2-nitro-phenyl)-carbamic acid tert.-butyl esters or (4-{alkenyl-, cycloalkenyl- or heterocycloalkenyl}-2-nitro-phenyl)-carbamic acid tert.-butyl esters by Stille-coupling of (2-nitro-4-tributylstannanyl-phenyl)-carbamic acid tert.-butyl ester with aryl halides or vinyl triflates or Stille-coupling of (4-iodo-2-nitrophenyl)-carbamic acid tert.-butyl ester with trialkylarylstannanes

A mixture of (2-nitro-4-tributylstannanyl-phenyl)-carbamic acid tert.-butyl ester (525 mg, 1.0 mmol; prepared

from the corresponding (4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Examples B) (10 mmol) by reaction with hexabutyldistannane (7.5 mL, 15 mmol) and $Pd(PPh_3)_4$ (116 mg, 0.1 mmol) in toluene (20 mL) at 60° C. for 5 days according to *Bull. Chem. Soc. Jpn.* 1983, 56, 3855–3856), aryl halide or vinyl triflate (0.95–6.0 mmol), anhydrous LiCl (126 mg, 3.0 mmol) and $Pd(PPh_3)_4$ (5 mol %) in DME (3 mL) was stirred at 100° C. under argon atmosphere until tlc indicated complete consumption of the stannane. The reaction was cooled to 23° C., stirred with sat. aqueous KF-sol. (5 mL) for 45 min, filtered through celite, washed with ether and the filtrate was dried over $MgSO_4$. Removal of the solvent in vacuum left a brown oil, which was purified by silica gel column chromatography with hexane/EtOAc to give the title compound.

The following examples relate to the preparation of (2-amino-4-arylethynyl-phenyl)-carbamic acid tert.-butyl esters in regioisomerically pure fashion (Synthetic Scheme G):

General Procedure K

Preparation of (4-alkynyl-2-nitro-phenyl)-carbamic acid tert.-butyl esters by Sonogashira-coupling of (4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl esters with acetylenic compounds

also Sonogashira-coupling of (4-ethynyl-2-nitro-phenyl)-carbamic acid tert.-butyl esters with aryl halides

and Sonogashira-coupling of 8-iodo-4-aryl-1,3-dihydro-benzo[b][1,4]diazepin-2-ones with acetylenic compounds

A mixture of the halide (3.0–4.5 mmol), acetylenic compound (3.0–4.5 mmol), Et_3N (13.5 mmol), $PdCl_2(PPh_3)_2$ (5 mol %) and PPh_3 (2.5 mol %) in THF (12 mL) [with very insoluble material DMF (up to 12 mL) could be added] was stirred for 20 min at 23° C. while being purged with Argon. CuI (1.2 mol %) was added and stirring was continued at 60° C. under Argon atmosphere until tlc indicated complete conversion of the minor component [cf. *J. Org. Chem.* 1998, 63, 8551]. The mixture was transferred into a separating funnel, 5% citric acid (50 mL) was added and the product was extracted with EtOAc (2×100 mL). The combined organic layers were washed with sat. $NaHCO_3$-sol. (50 mL) and brine (50 mL), followed by drying over $MgSO_4$. Removal of the solvent left a yellow residue, which was purified by silica gel column chromatography with hexane/EtOAc and/or triturated with hexane or aqueous EtOH to give the title compound.

Example K1

(5-Chloro-2-nitro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (5-chloro-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B1) (1.2 g, 3.0 mmol) and phenylacetylene (0.5 mL, 4.5 mmol) according to the general procedure K. Obtained as a yellow solid (944 mg).

MS (ISN) 371 [$(M-H)^-$] and 373 [$(M-H+2)^-$]; mp 166–167° C.

Example K2

(5-Methyl-2-nitro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (4-iodo-5-methyl-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B2) (1.13 g, 3.0 mmol) and phenylacetylene (0.5 mL, 4.5 mmol) according to the general procedure K. Obtained as a green-yellow solid (794 mg).

MS (EI) 352 (M^+); mp 161–164° C.

Example K3

[5-(4-Methyl-piperazin-1-yl)-2-nitro-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [4-iodo-5-(4-methyl-piperazin-1-yl)-2-nitro-phenyl]-carbamic acid tert.-butyl ester (Example C1) (1.34 g, 3.0 mmol) and phenylacetylene (0.5 mL, 4.5 mmol) according to the general procedure K. Obtained as a green-yellow solid (1.1 g).

MS (ISP) 437 [$(M+H)^+$]; mp 170° C.

Example K4

(5-Morpholin-4-yl-2-nitro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (4-iodo-5-morpholin-4-yl-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example C3) (890 mg, 2.0 mmol) and phenylacetylene (0.33 mL, 3.0 mmol) according to the general procedure K. Obtained as an orange solid (580 mg).

MS (ISP) 424 [$(M+H)^+$]; mp 190–191° C.

Example K5

(2-Nitro-4-phenylethynyl-5-thiomorpholin-4-yl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (4-iodo-2-nitro-5-thiomorpholin-4-yl-phenyl)-carbamic acid tert.-butyl ester (Example C2) (1.0 g, 2.15 mmol) and phenylacetylene (0.36 mL, 3.22 mmol) according to the general procedure K. Obtained as an orange solid (620 mg).

MS (ISP) 440 [$(M+H)^+$] and 462 [$(M+Na)^+$]; mp 201° C. (dec.).

Example K6

[5-(1,1-Dioxo-thiomorpholin-4-yl)-2-nitro-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared in two steps as followed:

To a solution of (4-iodo-2-nitro-5-thiomorpholin-4-yl-phenyl)-carbamic acid tert.-butyl ester (Example C2) (465 mg, 1 mmol) in acetone (25 mL) and H2O (1 mL) 0.3M ammoniummolybdate sol. (0.3 mL) and 33% H_2O_2 (2.3 mL) were added at 0° C. and mixture was stirred for 1 h at 23° C. Obtained the [5-(1,1-dioxo-thiomorpholin-4-yl)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester (497 mg, 1.0 mmol) as an amorphous yellow material, which was reacted with phenylacetylene (0.17 mL, 1.5 mmol) according to the general procedure K. Obtained as a yellow solid (245 mg).

MS (ISP) 472 [$(M+H)^+$]; mp 217–221° C.

Example K7

[5-(1,4-Dioxa-8-aza-spiro [4.5]dec-8-yl)-2-nitro-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [5-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester

(Example C4) (3.80 g, 7.53 mmol) and phenylacetylene (1.24 mL, 11.3 mmol) according to the general procedure K. Obtained as a orange solid (1.8 g).

MS (ISN) 478 [(M–H)$^-$]; mp 179–180° C.

Example K8

[5-(2-Dimethylamino-ethylsulfanyl)-2-nitro-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from (5-(2-dimethylamino-ethylsulfanyl)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester (Example D1) (721 mg, 1.54 mmol) and phenylacetylene (0.25 mL, 2.31 mmol) according to the general procedure K. Obtained as an amorphous yellow material (595 mg).

MS (ISP) 442 [(M+H)$^+$]; mp 179–180° C.

Example K9

(5-tert.-Butoxycarbonylamino-4-nitro-2-phenylethynyl-phenylsulfanyl)-acetic acid methyl ester

Prepared from (5-tert.-butoxycarbonylamino-2-iodo-4-nitro-phenylsulfanyl)-acetic acid methyl ester (Example D2) (780 mg, 1.67 mmol) and phenylacetylene (0.27 mL, 2.5 mmol) according to the general procedure K. Obtained as an orange solid (700 mg).

MS (ISP) 460 [(M+NH_4)$^+$]; mp 125–127° C.

Example K10

[5-(2-Methoxy-ethoxy)-2-nitro-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [4-iodo-5-(2-methoxy-ethoxy)-2-nitro-phenyl-carbamic acid tert.-butyl ester (Example B6) (876 mg, 2 mmol) and phenylacetylene (0.33 mL, 3 mmol) according to the general procedure K. Obtained as a yellow solid (569 mg).

MS (ISP) 413 [(M+H)$^+$] and 430 [(M+NH_4)$^+$]; mp 118–119° C.

Example K11

(5-Methoxy-2-nitro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (4-iodo-5-methoxy-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B5) (1.18 g, 3.00 mmol) and phenylacetylene (0.58 mL, 4.5 mmol) according to the general procedure K. Obtained as a yellow solid (1.1 g).

MS (EI) 368 (M$^+$); mp 129° C.

Example K12

[5-(2-{2-[2-(2-Methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-2-nitro-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from (4-iodo-5-(2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-2-nitro-phenyl]-carbamic acid tert.-butyl ester (Example B7) (5.7 g, 10.0 mmol) and phenylacetylene (1.65 mL, 15 mmol) according to the general procedure K. Obtained as a yellow oil (5.2 g).

MS (ISN) 543 [(M–H)$^-$].

Example K13

(5-tert.-Butoxycarbonylamino-4-nitro-2-phenylethynyl-phenoxy)-acetic acid tert.-butyl ester

Prepared from (5-tert.-butoxycarbonylamino-2-iodo-4-nitro-phenoxy)-acetic acid tert.-butyl ester (Example S1) (1.46 g, 2.99 mmol) and phenylacetylene (0.49 mL, 4.49 mmol) according to the general procedure K. Obtained as a yellow solid (1.4 g).

MS (ISP) 486 [(M+NH_4)$^+$]; mp 130° C.

Example K14

(RS)-5-(2,2-Dimethyl-[1,3]dioxolan-4-ylmethoxy)-2-nitro-4-phenylethynyl-phenylamine

Prepared from (RS)-5-(2,2-dimethyl-[1,3]dioxolan-4-ylmethoxy)-4-iodo-2-nitro-phenylamine (Example E5) (4.5 g, 11.4 mmol) and phenylacetylene (1.88 mL, 17.1 mmol) according to the general procedure K. Obtained as an orange solid (5.4 g).

MS (ISN) 367 [(M–H)$^-$]; mp 147–149 C.

Example K15

(5-tert.-Butoxycarbonylamino-4-nitro-2-phenylethynyl-benzoic acid methyl ester

Prepared from 5-tert.-butoxycarbonylamino-2-iodo-4-nitro-benzoic acid methyl ester (Example B3) (1.22 g, 2.89 mmol) and phenylacetylene (0.48 mL, 4.34 mmol) according to the general procedure K. Obtained as a yellow solid (793 mg).

MS (ISP) 397 [(M+H)$^+$] and 414 [(M+NH_4)$^+$]; mp 173° C.

Example K16

(5-tert.-Butoxycarbonylamino-4-nitro-2-phenylethynyl-phenyl)-acetic acid methyl ester

Prepared from (5-tert.-butoxycarbonylamino-2-iodo-4-nitro-phenyl)-acetic acid methyl ester (Example Ve (R=CO_2Me; R"=Boc)) (1.32 g, 3.03 mmol) and phenylacetylene (0.5 mL, 4.55 mmol) according to the general procedure K. Obtained as a yellow solid (1.1 g).

MS (ISP) 411 [(M+H)$^+$], 428 [(M+NH_4)$^+$] and 433 [(M+Na)$^+$]; mp 134° C.

Example K17

(5-Cyanomethyl-2-nitro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (5-cyanomethyl-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B9 [Ve (R=CN; R"=Boc)]) (3.97 g, 9.85 mmol) and phenylacetylene (3.24 mL, 29.56 mmol) according to the general procedure K. Obtained as an olive solid (1.6 g).

MS (ISP) 395 [(M+NH_4)$^+$]; mp 166° C.

Example K18

(RS)-[5-(2,3-Dihydroxy-propoxy)-2-nitro-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from (RS)-[5-(2,3-dihydroxy-propoxy)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester (RO-68-5451/000 (3.23 g, 7.11 mmol); prepared from (5-allyloxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B4) (4.20 g, 10.0 mmol) by reaction with NMO (1.28 g, 11.0 mmol), $OSO_4$2.5% in t-BuOH (1 mL, 0.1 mmol) and K_2OsO_4 (40 mg, 0.1 mmol) in acetone (250 mL) and H_2O (100 mL) at 23° C. for 6 days) and phenylacetylene

(1.17 mL, 10.67 mmol) according to the general procedure K. Obtained as a yellow solid (2.74 g).

MS (ISP) 429 [(M+H)$^+$]; mp 157° C. (dec.).

Example K19

(5-Hydroxymethyl-2-nitro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (5-hydroxymethyl-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example Vh) (2.61 g, 6.62 mmol) and phenylacetylene (1.10 mL, 9.93 mmol) according to the general procedure K. Obtained as a yellow solid (1.76 g).

MS (ISP) 386 [(M+NH_4)$^+$]; mp 177° C. (dec.).

Example K20

[5-(4-Methoxy-piperidin-1-yl)-2-nitro-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [4-iodo-5-(4-methoxy-piperidin-1-yl)-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example C5) (1.0 g, 2.1 mmol) and phenylacetylene (0.35 mL, 3.15 mmol) according to the general procedure K. Obtained as a yellow solid (799 mg).

mp 147–150° C.

Example K21

(5-Cyanomethoxy-2-nitro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (5-cyanomethoxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example S2) (605 mg, 1.44 mmol) and phenylacetylene (0.24 mL, 2.16 mmol) according to the general procedure K. Obtained as a yellow solid (508 mg).

MS (EI) 393 (M$^+$); mp 170° C.

Example K22

[4-(4-Fluoro-phenylethynyl)-5-hydroxymethyl-2-nitro-phenyl]-carbamic acid tert.-butyl ester

Prepared from (5-hydroxymethyl-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example Vh) (2.00 g, 5.07 mmol) and 4-fluorophenylacetylene (0.91 g, 7.61 mmol) according to the general procedure K. Obtained as a yellow solid (1.55 g).

MS (ISN) 385 [(M–H)$^-$]; mp 198° C.

Example K23

(RS)-(4-(4-Fluoro-phenylethynyl)-5-{methyl-[2-(tetrahydro-pyran-2-yloxy)-ethyl]-amino}-2-nitro-phenyl)-carbamic acid tert.-butyl ester

Prepared from (RS)-(4-iodo-5-{methyl-[2-(tetrahydro-pyran-2-yloxy)-ethyl]-amino}-2-nitro-phenyl)-carbamic acid tert-butyl ester [RO-69-3820/000, prepared from {5-[(2-hydroxy-ethyl)-methyl-amino]-4-iodo-2-nitro-phenyl}-carbamic acid tert.-butyl ester (Example C6) by treatment with 3,4-dihydro-2H-pyran and cat. TsOH.H_2O in DCM at 0° C.](2.09 g, 4.01 mmol) and 4-fluorophenylacetylene (0.72 g, 6.02 mmol) according to the general procedure K. Obtained as a yellow-brown solid (1.84 g).

MS (ISP) 514 [(M+H)$^+$]; mp 134° C.

Example K24

(RS)-{2-Nitro-4-phenylethynyl-5-[2-(tetrahydro-pyran-2-yloxy)-ethoxy]-phenyl}-carbamic acid tert.-butyl ester

Prepared from (RS)-{4-iodo-2-nitro-5-[2-(tetrahydro-pyran-2-yloxy)-ethoxy]-phenyl}-carbamic acid tert.-butyl ester (Example S3) (743 mg, 1.46 mmol) and phenylacetylene (0.24 mL, 2.19 mmol) according to the general procedure K. Obtained as a yellow-brown viscous oil (429 mg).

MS (EI) 393 (M$^+$).

Example K25

Prepared from [5-(2-tert.-butoxy-ethoxy)-4-iodo-2-nitro-phenyl]-carbamic acid tert.-butyl ester (Example B10) (1.44 g, 3.0 mmol) and 4-fluorophenylacetylene (541 mg, 4.5 mmol) according to the general procedure K. Obtained as a yellow solid (777 mg).

MS (EI) 472 (M$^+$); mp 96–98° C.

Example K26

[5-tert.-Butoxy-4-(4-fluoro-phenylethynyl)-2-nitro-phenyl]-carbamic acid tert.-butyl ester

Prepared from (5-tert.-butoxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example S4) (1.40 g, 3.21 mmol) and 4-fluorophenylacetylene (0.66 g, 5.46 mmol) according to the general procedure K. Obtained as a brown solid (520 mg).

MS (EI) 428 (M$^+$); mp 201° C.

General Procedure L

Preparation of the (2-amino-phenyl)-carbamic acid tert.-butyl esters by reduction of (2-nitro-phenyl)-carbamic acid tert.-butyl esters

Also preparation of 4-aryl-1,3-dihydro-benzo[b][1,4]diazepin-2-ones by reduction and concomitant cyclization of 3-aryl-N-(2-nitro-phenyl)-3-oxo-propionamides

Method a: Catalytic hydrogenation

A mixture of the nitro compound (1.0 mmol) in MeOH or EtOH and THF (1:1 ca. 20 mL) and 10% Palladium on carbon (20 mg) or Raney-Ni (20 mg) was stirred vigorously at 23° C. under hydrogen atmosphere until tlc indicated complete conversion. The catalyst was filtered off, washed thoroughly with MeOH or EtOH and THF (1:1), the solvent was removed in vacuum to give the title compound, which was generally pure enough for further transformations.

Method b: Reduction with $SnCl_2.2H_2O$

A mixture of the nitro compound (1.0 mmol) and $SnCl_2.2H_2O$ (5.0 mmol) was either stirred in EtOH (30 mL) at 70–80° C. or alternatively in pyridine (3 mL) and DMF (12 mL) at 23° C. under Argon atmosphere until tlc indicated complete conversion [cf. *Tetr. Lett.* 1984, 25, 839]. The reaction mixture was brought to pH 8 by addition of sat. $NaHCO_3$-sol. and extracted with EtOAc (2×100 mL). The combined organic layer were washed with brine and dried over Na_2SO_4. Removal of the solvent left a yellow solid, which—if necessary—can be purified by silica gel column chromatography.

Method c: Reduction with Zn and NH_4Cl

To a mixture of the nitro compound (1.0 mmol) in EtOH/THF/sat. NH_4Cl-sol. (1:1:1, 30 mL) was added Zinc dust (3.0 mmol) and the mixture was stirred at 70° C. under Argon atmosphere until tlc indicated complete conversion. Aqueous workup as described in method b.

Method d: Reduction with Fe and HOAc

To a mixture of the nitro compound (1.0 mmol) in THF/H_2O (4:1, 10–50 mL) was added Fe powder (6.0 mmol) and the mixture was stirred at 70° C. under Argon atmosphere until tlc indicated complete conversion. Aqueous workup as described in method b.

Example L1

(2-Amino-4-iodo-5-thiomorpholin-4-yl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (4-iodo-2-nitro-5-thiomorpholin-4-yl-phenyl)-carbamic acid tert.-butyl ester (Example C2) (1.05 g, 2.25 mmol) by reduction with $SnCl_2.2H_2O$ (2.54 g, 11.3 mmol) according to the general procedure L (method b). Obtained as a light yellow solid (993 mg).

MS (ISP) 436 [$(M+H)^+$]; mp 125–127° C.

Example L2

(2-Amino-4-iodo-5-morpholin-4-yl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (4-iodo-5-morpholin-4-yl-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example C3) (753 g, 1.65 mmol) by reduction with $SnCl_2.2H_2O$ (1.9 g, 8.27mmol) according to the general procedure L (method b). Obtained as a yellow solid (696 mg).

MS (ISP) 420 [$(M+H)^+$]; mp 139–143° C.

Example L3

(2-Amino-5-chloro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (5-chloro-2-nitro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester (Example K1) (742 mg, 2.0 mmol) by reduction with $SnCl_2.2\ H_2O$ (2.245 g, 10 mmol) according to the general procedure L (method b). Obtained as an orange solid (483 mg).

MS (ISP) 343 [$(M+H)^+$] and 345 [$(M+2+H)^+$].

Example L4

(2-Amino-5-methyl-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (5-methyl-2-nitro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester (Example K2) (741 mg, 2.1 mmol) by reduction with $SnCl_2.2H_2O$ (2.37 g, 10.3 mmol) according to the general procedure L (method b). Obtained as a light-brown solid (419 mg).

MS (ISP) 323 [$(M+H)^+$]; mp 172–173° C.

Example L5

[2-Amino-5-(4-methyl-piperazin-1-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [5-(4-methyl-piperazin-1-yl)-2-nitro-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example K3) (1.08 g, 2.48 mmol) by reduction with $SnCl_2.2H_2O$ (2.8 g, 12.4 mmol) according to the general procedure L (method b). Obtained as an orange solid (1.0 g).

MS (ISP) 407 [$(M+H)^+$]; mp 81–85° C.

Example L6

(5-Amino-2-chloro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (2-chloro-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example F1) (783 mg, 2.24 mmol) by reduction with $SnCl_2.2H_2$ O (2.53 g, 11.2 mmol) according to the general procedure L (method b). Obtained as a yellow solid (684 mg).

MS (EI) 318 (M^+) and 320 [$(M+2)^+$]; mp 109–111° C.

Example L7

(5-Amino-2-methyl-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (2-methyl-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example F2) (921 mg, 2.8 mmol) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a white solid (796 mg).

MS (EI) 298 (M^+); mp 122° C.

Example L8

[2-Amino-5-(2-dimethylamino-ethylsulfanyl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [5-(2-dimethylamino-ethylsulfanyl)-2-nitro-4-phenylethynyl -phenyl]-carbamic acid tert.-butyl ester (Example K8) (551 mg, 1.25 mmol) by reduction with $SnCl_2.2H_2O$ (1.41 g, 6.25 mmol) according to the general procedure L (method b). Obtained as an orange foam (510 mg).

MS (ISP) 412 [$(M+H)^+$]; mp 115–117° C.

Example L9

(4-Amino-5-tert.-butoxycarbonylamino-2-phenylethynyl-phenylsulfanyl)-acetic acid methyl ester

Prepared from (5-tert.-butoxycarbonylamino-4-nitro-2-phenylethynyl-phenylsulfanyl)-acetic acid methyl ester (Example K9) (634 mg, 1.43 mmol) by reduction with $SnCl_2.2H_2O$ (1.62 g, 7.16 mmol) according to the general procedure L (method b). Obtained as an orange gum (590 mg).

MS (ISP) 413 [$(M+H)^+$], 435 [$(M+Na)^+$] and 451 [$(M+K)^+$].

Example L10

(4-Amino-5-tert.-butoxycarbonylamino-2-phenylethynyl-phenyl)-acetic acid methyl ester

Prepared from (5-tert.-butoxycarbonylamino-4-nitro-2-phenylethynyl-phenyl)-acetic acid methyl ester (Example K16) (1.09 g, 2.66 mmol) by reduction with $SnCl_2.2H_2O$ (3.00 g, 13.28 mmol) according to the general procedure L (method b). Obtained as a light yellow solid (900 mg).

MS (ISP) 381 [$(M+H)^+$] and 403 [$(M+Na)^+$]; mp 130° C.

Example L11

[2-Amino-5-(2-methoxy-ethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [5-(2-methoxy-ethoxy)-2-nitro-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example K10) (1.417 g, 3.44 mmol) by reduction with $SnCl_2.2H_2O$ (3.88 g, 17.2 mmol) according to the general procedure L (method b). Obtained as an off-white solid (1.04 g).

MS (EI) 382 (M^+); mp 105–107° C.

Example L12

(2-Amino-5-morpholin-4-yl-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (5-morpholin-4-yl-2-nitro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester (Example K4) (563 mg, 1.329 mmol) by reduction with $SnCl_2.2H_2O$ (1.5 g, 6.65 mmol) according to the general procedure L (method b). Obtained as a red-brown solid (488 mg).

MS (ISP) 394 [$(M+H)^+$]; mp 174–176° C.

Example L13

(2-Amino-5-methoxy-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (5-methoxy-2-nitro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester Example K11) (1.00 g, 2.71 mmol) by reduction with $SnCl_2.2H_2O$ (3.06 g, 13.57 mmol) according to the general procedure L (method b). Obtained as a yellow solid (870 mg).

MS (EI) 338 (M^+); mp 158° C.

Example L14

4-Amino-5-tert.-butoxycarbonylamino-2-phenylethynyl-benzoic acid methyl ester

Prepared from (5-tert.-butoxycarbonylamino-4-nitro-2-phenylethynyl-benzoic acid methyl ester (Example K15) (754 mg, 1.90 mmol) by reduction with $SnCl_2.2H_2O$ (2.15 g, 9.51 mmol) according to the general procedure L (method b). Obtained as a pink solid (431 mg).

MS (EI) 366 (M^+); mp 164° C.

Example L15

[2-Amino-5-(2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [5-(2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-2-nitro-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example K12) (3.0 g, 5.51 mmol) by reduction with $SnCl_2.2H_2O$ (6.2 g, 27.54 mmol) according to the general procedure L (method b). Obtained as a brown oil (2.9 g).

MS (ISP) 515 [$(M+H)^+$].

Example L16

(4-Amino-5-tert.-butoxycarbonylamino-2-phenylethynyl-phenoxy)-acetic acid tert.-butyl ester

Prepared from (5-tert.-butoxycarbonylamino-4-nitro-2-phenylethynyl-phenoxy)-acetic acid tert.-butyl ester (Example K13) (1.32 g, 2.82 mmol) by reduction with $SnCl_2.2H_2O$ (3.18 g, 14.10 mmol) according to the general procedure L (method b). Obtained as an amorphous orange material (1.2 g).

MS (ISP) 439 [$(M+H)^+$] and 461 [$(M+Na)^+$].

Example L17

(2-Amino-5-cyanomethyl-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (5-cyanomethyl-2-nitro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester (Example K17) (377 mg, 1.0 mmol) by reduction with $SnCl_2.2H_2O$ (1.13 g, 5.0 mmol) according to the general procedure L (method b). Obtained as an orange solid (338 mg).

MS (ISP) 348 [$(M+H)^+$] and 370 [$(M+Na)^+$]; mp 143° C.

Example L18

[2-Amino-5-(1,4-dioxa-8-aza-spiro [4.5]dec-8-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester and [2-Amino-5-(4,4-diethoxy-piperidin-1-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [5-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-2-nitro-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example K7) (1.73 g, 3.6 mmol) by reduction with $SnCl_2.2H_2O$ (4.0 g, 18.0 mmol) in EtOH according to the general procedure L (method b). Obtained as a brown solid (418 mg) and as dark brown solid (379 mg), respectively.

MS (ISP) 450 [$(M+H)^+$]; mp 79–82° C.; MS (ISP) 480 [$(M+H)^+$].

Example L19

[2-Amino-5-(1,1-dioxo-6-thiomorpholin-4-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [5-(1,1-dioxo-thiomorpholin-4-yl)-2-nitro-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example K6) (235 mg, 0.5 mmol) by reduction with $SnCl_2.2H_2O$ (564 mg, 2.5 mmol) according to the general procedure L (method b). Obtained as a brown solid (418 mg, impure material, used directly in Example 6 without purification and characterization).

Example L20

[2-Amino-5-(2,2-dimethyl-[1,3]dioxolan-4-ylmethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from (RS)-[5-(2,2-dimethyl-[1,3]dioxolan-4-ylmethoxy)-2-nitro-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example B8) (1.90 g, 4.06 mmol) by reduction with $SnCl_2.2H_2O$ (4.6 g, 20.32 mmol) according to the general procedure L (method b). The crude product was reprotected by stirring with 2,2-dimethoxypropane (5 mL) and p-TsOH.H_2O (1.1 eq) in DMF (5 mL) at 23° C. for 4 h. Obtained as a brown solid (1.1 g).

MS (ISP) 439 [$(M+H)^+$], 461 [$(M+Na)^+$] and 477 [$(M+K)^+$].

Example L21

4-Amino-5-tert.-butoxycarbonylamino-2-iodo-benzoic acid methyl ester

Prepared from 5-tert.-butoxycarbonylamino-2-iodo-4-nitro-benzoic acid methyl ester (Example B3) (3.00 g, 7.11 mmol) by reduction with $SnCl_2.2H_2O$ (8.02 g, 35.55 mmol) according to the general procedure L (method b). Obtained as a light red foam (1.9 g).

MS (ISP) 393 [$(M+H)^+$]; mp 60–78° C.

Example L22

[RS]-[2-Amino-5-(2-oxo-[1,3]dioxolan-4-ylmethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [RS]-[2-Nitro-5-(2-oxo-[1,3]dioxolan-4-ylmethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-

butyl ester [RO-68-8108/000 (411 mg, 0.9 mmol), prepared from (RS)-[5-(2,3-dihydroxy-propoxy)-2-nitro-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example K18) by treatment with 1,1'-carbonyldiimidazole in THF at 0 to 23° C.] by reduction with $SnCl_2.2H_2O$ (1.02 g, 4.5 mmol) according to the general procedure L (method b). Obtained as an apricot solid (370 mg).

MS (ISP) 425 [$(M+H)^+$]; mp 140° C.

Example L23

(2-Amino-5-ethoxymethyl-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester

Prepared from carbonic acid 5-tert.-butoxycarbonylamino-4-nitro-2-phenylethynyl-benzyl ester methyl ester [RO-68-8481/000, prepared from (5-hydroxymethyl-2-nitro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester (Example K19) by treatment with methyl chloroformate and Et_3N in THF at 0° C.] by reduction with $SnC_2.2H_2O$ according to the general procedure L (method b). Obtained as an amorphous brown substance (139 mg).

MS (ISP) 367 [$(M+H)^+$].

Example L24

2,2-Dimethyl-propionic acid 4-amino-5-tert.-butoxycarbonylamino-2-phenylethynyl-benzyl ester

Prepared from 2,2-dimethyl-propionic acid 5-tert-butoxycarbonylamino-4-nitro-2-phenylethynyl-benzyl ester [RO-68-9779/000, prepared from (5-hydroxymethyl-2-nitro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester (Example K19) by treatment with pivaloyl chloride and cat. DMAP in pyridine at 0 to 23° C.] by reduction with $SnCl_2.2H_2O$ according to the general procedure L (method b). Obtained as an amorphous yellow substance (182 mg).

MS (ISN) 421 [$(M-H)^-$].

Example L25

(RS)-2-Amino-4-phenylethynyl-5-(tetrahydro-pyran-2-yloxymethyl)-phenyl]-carbamic acid tert.-butyl ester

Prepared from (RS)-[2-nitro-4-phenylethynyl-5-(tetrahydro-pyran-2-yloxymethyl)-phenyl]-carbamic acid tert-butyl ester [RO-69-2829/000, prepared from (5-hydroxymethyl-2-nitro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester (Example K19) by treatment with 3,4-dihydro-2 H-pyran and cat. $TsOH.H_2O$ in DCM at 0° C.] by reduction with $SnCl_2.2H_2O$ according to the general procedure L (method b). Obtained as a yellow solid (1.78 g).

MS (ISN) 421 [$(M-H)^-$]; mp 158° C.

Example L26

[2-Amino-5-(4-methoxy-piperidin-1-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [5-(4-methoxy-piperidin-1-yl)-2-nitro-4-phenylethynyl-phenyl-carbamic acid tert.-butyl ester (Example K20) (727 mg, 1.61 mmol) by reduction with $SnC_2.2H_2O$ according to the general procedure L (method b). Obtained as a yellow solid (489 mg).

MS (ISP) 422 [$(M+H)^+$]; mp 173–176° C.

Example L27

(2-Amino-5-cyanomethoxy-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester

Prepared from (5-cyanomethoxy-2-nitro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester (Example K21) (395 mg, 0.91 mmol) by reduction with $SnCl_2.2H_2O$ according to the general procedure L (method b). Obtained as an amorphous yellow substance (219 mg).

MS (ISP) 364 [$(M+H)^+$].

Example L28

(RS)-[2-Amino-4-(4-fluoro-phenylethynyl)-5-(tetrahydro-pyran-2-yloxymethyl)-phenyl]-carbamic acid tert.-butyl ester

Prepared from (RS)-[4-(4-fluoro-phenylethynyl)-2-nitro-5-(tetrahydro-pyran-2-yloxymethyl)-phenyl]-carbamic acid tert.-butyl ester [RO-69-3877/000, prepared from [4-(4-fluoro-phenylethynyl)-5-hydroxymethyl-2-nitro-phenyl]-carbamic acid tert.-butyl ester (Example K22) by treatment with 3,4-dihydro-2 H-pyran and cat. $TsOH.H_2O$ in DCM at 0° C.] by reduction with $SnC_2.2H_2O$ according to the general procedure L (method b). Obtained as an amorphous light brown substance (990 mg).

MS (ISP) 441 [$(M+H)^+$].

Example L29

(RS)-(2-Amino-4-(4-fluoro-phenylethynyl)-5-{methyl-[2-(tetrahydro-pyran-2-yloxy)-ethyl]-amino}-phenyl)-carbamic acid tert.-butyl ester

Prepared from (RS)-(4-(4-fluoro-phenylethynyl)-5-{methyl-[2-(tetrahydro-pyran-2-yloxy)-ethyl]-amino}-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example K23) (1.79 g, 3.49 mmol) by reduction with $SnCl_2.2H_2O$ according to the general procedure L (method b). Obtained as an amorphous light brown substance (1.20 g).

MS (ISP) 484 [$(M+H)^+$].

Example L30

(RS)-{2-Amino-4-phenylethynyl-5-[2-(tetrahydro-pyran-2-yloxy)-ethoxy]-phenyl }-carbamic acid tert.-butyl ester

Prepared from (RS)-{2-nitro-4-phenylethynyl-5-[2-(tetrahydro-pyran-2-yloxy)-ethoxy]-phenyl}-carbamic acid tert.-butyl ester (Example K24) (420 mg, 0.87 mmol) by reduction with $SnCl_2.2H_2O$ according to the general procedure L (method b). Obtained as a light brown solid (346 mg).

MS (ISP) 453 [$(M+H)^+$].

Example L31

(RS)-{5-Amino-4'-fluoro-2-[4-(tetrahydro-pyran-2-yloxy)-piperidin-1-yl]-biphenyl-4-yl}carbamic acid tert.-butyl ester

Prepared from (RS)-{4'-fluoro-5-nitro-2-4-(tetrahydro-pyran-2-yloxy)-piperidin-1-yl]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example F3) (845 mg, 1.64 mmol) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a light green solid (758 mg).

MS (ISP) 486 [$(M+H)^+$]; mp 157–161° C.

Example L32

[2-Amino-5-(2-tert.-butoxy-ethoxy)-4-(4-fluoro-phenylethynyl)-phenyl]-carbamic acid tert.-butyl ester

Prepared from [5-(2-tert.-butoxy-ethoxy)-4-(4-fluoro-phenylethynyl)-2-nitro-phenyl]-carbamic acid tert.-butyl ester (Example K25) (744 mg, 1.57 mmol) by reduction with $SnCl_2.2H_2O$ according to the general procedure L (method b). Obtained as a light yellow solid (575 mg).

MS (ISP) 443 [(M+H)$^+$]; mp 149–150 C.

Example L33

(RS)-(5-Amino-4'-fluoro-2-{4-[2-(tetrahydro-pyran-2-yloxy)-ethoxy]-piperidin-1-yl}-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (RS)-(4'-fluoro--nitro-2-{4-[2-(tetrahydro-pyran-2-yloxy)-ethoxy]-piperidin-1-yl}-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example F4) (900 mg, 1.61 mmol) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a light brown foam (779 mg).

MS (ISP) 530 [(M+H)$^+$]; mp 56–58° C.

Example L34

(RS)-[5-Amino-4'-fluoro-2-(tetrahydro-pyran-2-yloxymethyl)-biphenyl-4-yl -carbamic acid tert-butyl ester

Prepared from (RS)-[4'-fluoro-5-nitro-2-(tetrahydro-pyran-2-yloxymethyl)-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example F5) by reduction with $SnCl_2.2H_2O$ according to the general procedure L (method b). Obtained as an orange solid (1.15 g).

mp 139–142° C.

Example L35

(5-Amino-2-cyanomethoxy-4'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (2-cyanomethoxy-4'-fluoro-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example F6) (310 mg, 0.8 mmol) by reduction with $SnCl_2.2H_2O$ according to the general procedure L (method b). Obtained as a light brown solid (220 mg).

MS (ISN) 356 [(M−H)$^-$]; mp 118–119° C.

Example L36

(5-Amino-2-dimethylaminomethyl-4'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (2-dimethylaminomethyl-4'-fluoro-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example F7) by reduction with $SnCl_2.2H_2O$ according to the general procedure L (method b). Obtained as a yellow solid (908 mg).

mp 97–125° C.

Example L37

[5-Amino-2-(2,2-dimethyl-tetrahydro-[1,3]dioxolo [4,5-c]pyrrol-5-yl)-4'-fluoro-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [2-(2,2-dimethyl-tetrahydro-(1,3]dioxolo [4,5-c]pyrrol-5-yl)-4'-fluoro-5-nitro-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example F8) (610 mg, 1.29 mmol) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as an off-white foam (578 mg).

MS (ISP) 444 [(M+H)$^+$].

Example L38

(5-Amino-4'-fluoro-2-methoxy-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (4'-fluoro-2-methoxy-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example F9) (2.64 g, 7.29 mmol) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as an off-white solid (2.36 g).

MS (ISP) 333 [(M+H)$^+$]; mp 155° C. (dec.).

Example L39

[5-Amino-2-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-4'-fluoro-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [2-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-4'-fluoro-5-nitro-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example F10) (2.4 g, 5.0 mmol) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a green solid (2.37 g).

MS (ISP) 444 [(M+H)$^+$].

Example L40

(5-Amino-4'-fluoro-2-methyl-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (4'-fluoro-2-methyl-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example F11) (560 mg, 1.62 mmol) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a light brown solid (512 mg).

MS (ISP) 317 [(M+H)$^+$]; mp 112° C.

Example L41

(5-Amino-4-tert.-butoxycarbonylamino-4'-fluoro-biphenyl-2-yloxy)-acetic acid tert.-butyl ester

Prepared from (4-tert.-butoxycarbonylamino-4'-fluoro-5-nitro-biphenyl-2-yloxy)-acetic acid tert.-butyl ester (Example F12) (2.29 g, 4.95 mmol) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a dark blue solid (2.14 g).

MS (ISP) 433 [(M+H)$^+$]; mp 30–33° C.

Example L42

(5-Amino-2-chloro-4'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (2-chloro-4'-fluoro-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example F13) by reduction with $SnCl_2.2H_2O$ according to the general procedure L (method b). Obtained as a light red solid (544 mg).

MS (ISP) 337 [(M+H)$^+$].

Example L43

[5-Amino-4'-fluoro-2-(2-methoxy-ethoxy)-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [4'-fluoro-2-(2-methoxy-ethoxy)-5-nitro-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example

F14) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a light brown solid (1.652 g).

MS (ISP) 377 [(M+H)$^+$].

Example L44

[5-Amino-2-(2-tert.-butoxy-ethoxy)-4'-fluoro-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [2-(2-tert.-butoxy-ethoxy)-4'-fluoro-5-nitro-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example F15) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a purple solid (547 mg).

MS (ISP) 419 [(M+H)^{30}]; mp 133° C. (dec.).

Example L45

[5-Amino-4'-fluoro-2-(2-oxo-oxazolidin-3-yl)-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [4'-fluoro-5-nitro-2-(2-oxo-oxazolidin-3-yl)-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example F16) (280 mg, 0.67 mmol) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a yellow solid (277 mg).

MS (ISP) 388 [(M+H)$^+$]; mp 210° C.

Example L46

(5-Amino-4'-fluoro-2-methoxy-2'-methyl-biphenyl-4-y)-carbamic acid tert.-butyl ester

Prepared from (4'-fluoro-2-methoxy-2'-methyl-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example F17) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a brown solid (588 mg).

MS (ISP) 347 [(M+H)$^+$].

Example L47

(5-Amino-2-tert.-butoxy-4'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (2-tert.-butoxy-4'-fluoro-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example F18) (1.15 g, 2.84 mmol) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a pink solid (747 mg).

MS (ISP) 375 [(M+H)$^+$]; mp 139° C.

Example L48

(5-Amino-2-tert.-butoxy-2'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (2-tert.-butoxy-2'-fluoro-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example F19) (930 mg, 2.3 mmol) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a pink solid (649 mg).

MS (ISP) 375 [(M+H)$^+$]; mp 130° C.

Example L49

(RS)-{5-Amino-4'-fluoro-2-[(R)-3-(tetrahydro-pyran-2-yloxy)-pyrrolidin-1-yl]-biphenyl -4-yl}-carbamic acid tert.-butyl ester

Prepared from (RS)-{4'-fluoro-5-nitro-2-[(R)-3-(tetrahydro-pyran-2-yloxy)-pyrrolidin-1-yl]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example F20) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a dark green solid (852 mg).

MS (ISP) 472 [(M+H)$^+$].

Example L50

(5-Amino-2'-fluoro-2-methoxy-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (2'-fluoro-2-methoxy-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example F21) (649 mg, 1.79 mmol) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a light brown solid (352 mg).

MS (ISP) 333 [(M+H)$^+$]; mp 161° C.

Example L51

[5-Amino-2-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-2'-fluoro-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [2-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-2'-fluoro-5-nitro-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example F22) (1.39 g, 2.94 mmol) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a light beige solid (1.01 g).

MS (ISP) 444 [(M+H)$^+$]; mp 198° C.

Example L52

(5-Amino-2',5'-difluoro-2-methoxy-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (2',5'-difluoro-2-methoxy-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example F23) (1.05 g, 2.76 mmol) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a beige solid (618 mg).

MS (ISN) 349 [(M−H)$^-$]; mp 144° C.

Example L53

[5-Amino-2'-fluoro-2-(2-methoxy-ethoxy)-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [2'-fluoro-2-(2-methoxy-ethoxy)-5-nitro-biphenyl-4-yl]carbamic acid tert.-butyl ester (Example F24) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as a purple solid (2.581 g).

Example L54

(RS)-[5-Amino-2'-fluoro-2-(tetrahydro-pyran-2-yloxymethyl)-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from (RS)-[2'-fluoro-5-nitro-2-(tetrahydro-pyran-2-yloxymethyl)-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example F25) by reduction with $SnCl_2.2H_2O$ according to the general procedure L (method b). Obtained as a yellow liquid (2.676 g).

MS (ISP) 439 [(M+Na)$^+$].

Example L55

[5-Amino-2'-fluoro-2-(4-methoxy-benzyloxy)-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [2'-fluoro-2-(4-methoxy-benzyloxy)-5-nitro-biphenyl-4-yl]-carbamic acid tert.-butyl ester

(Example F25) (0.90 g, 1.92 mmol) by reduction with $SnCl_2.2H_2O$ according to the general procedure L (method b). Obtained as a beige solid (719 mg).

MS (ISP) 439 [$(M+H)^+$].

Example L56

(5-Amino-2-tert.-butoxy-2',5'-difluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (2-tert.-butoxy-2',5'-difluoro-5-nitro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example F27) by catalytic hydrogenation with Pd/C according to the general procedure L (method a). Obtained as an amorphous grey-blue substance (2.37 g).

MS (ISP) 393 [$(M+H)^+$].

Example L57

[2-Amino-5-tert.-butoxy-4-(4-fluoro-phenylethynyl)-phenyl]-carbamic acid tert.-butyl ester

Prepared from [5-tert.-butoxy-4-(4-fluoro-phenylethynyl)-2-nitro-phenyl]-carbamic acid tert.-butyl ester (Example K26) (649 mg, 1.51 mmol) by reduction with $SnCl_2.2H_2O$ according to the general procedure L (method b). Obtained as a light yellow solid (410 mg).

MS (ISP) 399 [$(M+H)^+$]; mp 183° C.

The following examples relate to the preparation of the ethyl or tert.-butyl 3-aryl-3-oxo-propionates (formula VIIa), which serve as building blocks in the synthesis of the target compounds (Synthetic Scheme H):

General Procedure M

Method a) Preparation of ethyl or tert.-butyl 3-aryl-3-oxo-propionates

The ethyl or tert.-butyl 3-aryl-3-oxo-propionates were prepared from the aryl acid chlorides and ethyl or tert.-butyl malonate potassium salt [CAS-no. 6148-64-7 and 75486-33-8] with Et_3N and $MgCl_2$ in CH_3CN at 0° C. to 23° C. according to *Synthesis* 1993, 290. If the carboxylic acid was employed in this reaction, it was activated by treatment with ethyl chloroformate and Et_3N in THF/CH_3CN at 0° C. prior to reaction with the malonate salt.

Method b) Preparation of tert.-butyl 3-aryl-3-oxo-propionates

The tert.-butyl 3-aryl-3-oxo-propionates were alternatively prepared from the methyl or ethyl aryl esters by treatment with lithium tert.-butyl acetate [prepared by treatment of tert.-butyl acetate with lithium diisopropylamide in THF at −78° C.] in the presence of lithium tert.-butoxide according to *Synthesis* 1985, 45. If the product contained residual starting material after workup, thus could be removed by selective saponification with LiOH in THF/MeOH/H_2O at 23° C.

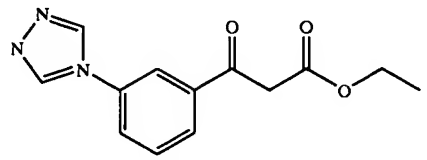
Method c) Preparation of 3-aryl-3-oxo-propionic acids

The 3-aryl-3-oxo-propionic acids were prepared from the aryl acid chlorides and bis(trimethylsilyl)malonate with Et_3N and LiBr in CH_3CN at 0° C. according to *Synth. Commun.* 1985, 15, 1039 (method c1) or with n-BuLi in ether at −60° C. to 0° C. according to *Synthesis* 1979, 787 (method c2).

Example M1

3-Oxo-3-(3-[1,2,4]triazol-4-yl-phenyl)-propionic acid ethyl ester

RO-71-2790/000



Prepared from 3-[1,2,4]triazol-4-yl-benzoic acid [RO-71-1432/000, prepared by reaction of 3-aminobenzoic acid with hydrazine hydrate and triethyl orthoformate in acetic acid at 120° C.] by activation with ethyl chloroformate/Et_3N and reaction with ethyl malonate potassium salt with Et_3N and $MgCl_2$ in CH_3CN according to general procedure M (method a). Obtained as a white solid (5.74 g).

MS (EI) 259 (M^+).

Example M2

3-Oxo-3-(3-[1,2,3]triazol-1-yl-phenyl)-propionic acid ethyl ester

Prepared from 3-[1,2,3]triazol-1-yl-benzoic acid [RO-71-3703/000, prepared by refluxing of methyl 3-azidobenzoate [CAS-No. 93066-93-4] in trimethylsilylacetylene, followed by saponification with aqueous NaOH in refluxing EtOH] by activation with ethyl chloroformate/Et_3N and reaction with ethyl malonate potassium salt with Et_3N and $MgCl_2$ in CH_3CN according to general procedure M (method a). Obtained as a light yellow solid (2.22 g).

MS (EI) 259 (M^+); mp 72–74° C.

Example M3

3-(3-Cyano-phenyl)-3-oxo-propionic acid tert.-butyl ester

Prepared from methyl 3-cyanobenzoate [CAS-No. 13531-48-1] by treatment with lithium tert.-butyl acetate according to general procedure M (method b). Obtained as a light brown oily semisolid.

MS (EI) 245 (M^+).

Example M4

3-(3-Imidazol-1-yl-phenyl)-3-oxo-propionic acid tert.-butyl ester

Prepared from methyl 3-(1H-imidazol-1-yl)benzoate [prepared from 3-(1H-imidazol-1-yl)benzoic acid (*J. Med. Chem.* 1987, 30, 1342; CAS-No. [108035-47-8] by refluxing in conc. H_2SO_4/MeOH] by treatment with lithium tert.-butyl acetate according to general procedure M (method b). Obtained as an orange-brown oil.

MS (ISP) 287 [$(M+H)^+$].

Example M5

3-(2-Imidazol-1-yl-pyridin-4-yl)-3-oxo-propionic acid tert.-butyl ester

Prepared from 2-imidazol-1-yl-isonicotinoyl chloride hydrochloride [prepared by reaction of tert.-butyl

2-chloroisonicotinoate with imidazole and NaH in DMF at 80° C., treatment with formic acid at 50° C. and reaction with thionylchloride in toluene at 100° C.] and tert.-butyl malonate potassium salt with Et_3N and $MgCl_2$ in CH_3CN according to general procedure M (method a). Obtained as a brown solid (10.8 g).

MS (EI) 287 (M^+); mp 80° C. (dec.).

Example M6

3-Oxo-3-(3-[1,2,4]triazol-1-yl-phenyl)-propionic acid tert.-butyl ester

Prepared from methyl 3-[1,2,4]triazol-1-yl-benzoate [CAS-No. 167626-27-9] by treatment with lithium tert.-butyl acetate according to general procedure M (method b). Obtained as an orange liquid (2.41 g).

MS (EI) 287 (M^+).

Example M7

3-[3-(4-Methyl-imidazol-1-yl)-phenyl]-3-oxo-propionic acid tert.-butyl ester

Prepared from methyl 3-(4-methyl-imidazol-1-yl)-benzoate [RO-69-6483/000, prepared the corresponding acid from 3-isothiocyanatobenzoic acid and 2-aminopropionaldehyde dimethyl acetal according to *J. Med. Chem.* 1987, 30, 1342, followed by refluxing in conc. H_2SO_4/MeOH] by treatment with lithium tert.-butyl acetate according to general procedure M (method b). Obtained as a yellow-brown oil (10.69 g).

MS (EI) 300 (M^+).

Example M8

3-[3-(2-Methyl-imidazol-1-yl)-phenyl]-3-oxo-propionic acid tert.-butyl ester

Prepared from ethyl 3-(2-methyl-imidazol-1-yl)-benzoate [RO-69-7480/000, prepared by reaction of ethyl 3-aminobenzoate with ethyl acetimidate hydrochloride in EtOH at 0° C., direct treatment with aminoacetaldehyde diethyl acetal in EtOH at 23° C., follwed by addition of conc. H_2SO_4 and refluxing.] by treatment with lithium tert.-butyl acetate according to general procedure M (method b). Obtained as a brown oil (9.66 g).

MS (ISN) 299 [$(M-H)^-$].

Example M9

3-[3-(2,4-Dimethyl-imidazol-1-yl)-phenyl]-3-oxo-propionic acid tert.-butyl ester

Prepared from ethyl 3-(2,4-dimethyl-imidazol-1-yl)-benzoate [RO-71-0583/000, prepared by reaction of ethyl 3-aminobenzoate with ethyl acetimidate hydrochloride in EtOH at 0° C., direct treatment with 2-aminopropionaldehyde dimethyl acetal in EtOH at 23° C., follwed by addition of conc. H_2SO_4 and refluxing.] by treatment with lithium tert.-butyl acetate according to general procedure M (method b). Obtained as a yellow-brown oil (6.00 g).

MS (ISN) 313 [$(M-H)^-$].

Example M10

3-(2-Cyano-pyridin-4-yl)-3-oxo-propionic acid tert.-butyl ester

Prepared from 2-cyano-isonicotinic acid ethyl ester [CAS-No. 58481-14-4] by treatment with lithium tert.-butyl acetate according to general procedure M (method b). Obtained as a light brown solid (7.70 g).

MS (ISN) 245 [$(M-H)^-$].

Example M11

3-Oxo-3-(3-[1,2,4]triazol-4-yl-phenyl)-propionic acid tert.-butyl ester

Prepared from methyl 3-[1,2,4]triazol-4-yl-benzoate [prepared by reaction of 3-aminobenzoic acid with hydrazine hydrate and triethyl orthoformate in acetic acid at 120° C., followed by esterification with conc. H_2SO_4 in refluxing MeOH] by treatment with lithium tert.-butyl acetate according to general procedure M (method b). Obtained as a light yellow gum (870 mg).

MS (ISN) 286 [$(M-H)^-$].

Example M12

3-[3-(2-Methoxymethylsulfanyl-imidazol-1-yl)-phenyl]-3-oxo-propionic acid tert.-butyl ester

Prepared from ethyl 3-(2-methoxymethylsulfanyl-imidazol-1-yl)-benzoate [prepared by esterification of 3-(2-methoxymethylsulfanyl-imidazol-1-yl)-benzoic acid [CAS-No. 108035-46-7] with conc. H_2SO_4 in EtOH, followed by treatment with chloromethylmethyl ether and NaH in THF/DMF] by treatment with lithium tert.-butyl acetate according to general procedure M (method b). Obtained as an orange oil (1.82 g).

MS (EI) 362 (M^+).

Example M13

3-[3-(2-Methylsulfanyl-imidazol-1-yl)-phenyl]-3-oxo-propionic acid tert.-butyl ester

Prepared from ethyl 3-(2-methylsulfanyl-imidazol-1-yl)-benzoate [prepared by esterification of 3-(2-methoxymethylsulfanyl-imidazol-1-yl)-benzoic acid [CAS-No. 108035-46-7] with conc. H_2SO_4 in EtOH, followed by treatment methyl iodide and NaH in THF/DMF] by treatment with lithium tert.-butyl acetate according to general procedure M (method b). Obtained as a light brown oil (4.41 g).

MS (ISP) 333 [$(M+H)^+$].

Example M 14

3-[3-(3-Methyl-isoxazol-5-yl)-phenyl]-3-oxo-propionic acid tert.-butyl ester

Prepared from ethyl 3-(3-methyl-isoxazol-5-yl)-benzoate [prepared by reaction of ethyl 3-ethynylbenzoate [CAS-No. 178742-95-5] with a mixture of NCS, acetaldoxime, Et_3N and cat. amount of pyridine in $CHCl_3$ at 50° C. according to *Tetrahedron* 1984, 40, 2985–2988] by treatment with lithium tert.-butyl acetate according to general procedure M (method b). Obtained as a yellow solid (2.54 g).

MS (ISP) 302 [$(M+H)^+$]; mp 50–56° C.

Example M15

3-Oxo-3-(3-tetrazol-1-yl-phenyl)-propionic acid ethyl ester

Prepared from 3-tetrazol-1-yl-benzoic acid [CAS-No. 204196-80-5] by activation with ethyl chloroformate/Et_3N

and reaction with ethyl malonate potassium salt with Et_3N and $MgCl_2$ in CH_3CN according to general procedure M (method a). Obtained as a light yellow solid (211 mg).

MS (EI) 260 (M^+).

Example M16

3-(3-Chloro-thiophen-2-yl)-3-oxo-propionic acid ethyl ester

Prepared from 3-chloro-2-thiophenecarbonyl chloride [CAS-No. 86427-02-3] by reaction with ethyl malonate potassium salt with Et_3N and $MgCl_2$ in CH_3CN according to general procedure M (method a). Obtained as a brown oil (6.84 g).

MS (EI) 232 (M^+) and 234 [$(M+2)^+$].

Example M17

3-(5-Cyano-thiophen-2-yl)-3-oxo-propionic acid tert.-butyl ester

Prepared from ethyl 5-cyano-2-thiophenecarboxylate [CAS-No. 67808-35-9] by treatment with lithium tert.-butyl acetate according to general procedure M (method b). Obtained as a yellow solid (6.66 g).

MS (EI) 251 (M^+); mp 78° C.

Example M18

3-(5-Cyano-2-fluoro-phenyl)-3-oxo-propionic acid ethyl ester

Prepared from 5-cyano-2-fluoro-benzoyl chloride [prepared from the corresponding acid [CAS-No. 146328-87-2] by treatment with $SOCl_2$, cat. DMF in toluene at 80° C.] by reaction with ethyl malonate potassium salt with Et_3N and $MgCl_2$ in CH_3CN according to general procedure M (method a). Obtained as a light yellow solid (3.85 g).

MS (EI) 235 (M^+); mp 55–60° C.

Example M19

3-(2-Imidazol-1-yl-thiazol-4-yl)-3-oxo-propionic acid tert.-butyl ester

Prepared from ethyl 2-imidazol-1-yl-thiazole-4-carboxylate [CAS-No. 256420-32-3] by treatment with lithium tert.-butyl acetate according to general procedure M (method b). Obtained as an orange oil (12.0 g).

Example M20

3-[2-(4-Methyl-imidazol-1-yl)-thiazol-4-yl]-3-oxo-propionic acid tert.-butyl ester

Prepared from ethyl 2-(4-methyl-imidazol-1-yl)-thiazole-4-carboxylate [prepared from ethyl 2-amino-4-thiazolecarboxylate (CAS-No. [256420-32-3]) by the following synthetic sequence: 1.) NaH, 2-isothiocyanato-1,1-dimethoxy-propane, DMF, 23° C.; 2.) aq. H_2SO_4, reflux; 3.) EtOH, conc. H_2SO_4, 23° C.; 4.) 30% H_2O_2, HOAc, 23° C.] by treatment with lithium tert.-butyl acetate according to general procedure M (method b). Obtained as a brown oil (8.73 g).

MS (EI) 307 (M^+).

Example M21

3-[3-(1-Methyl-1H-imidazol-2-yl)-phenyl]-3-oxo-propionic acid tert.-butyl ester

Prepared from ethyl 3-(1-methyl-1H-imidazol-2-yl) benzoate [CAS-No. 168422-44-4] by treatment with lithium tert.-butyl acetate according to general procedure M (method b). Obtained as a light yellow liquid (1.26 g).

MS (ISP) 301.3 [$(M+H)^+$].

The following examples relate to the preparation of the 6-aryl-2,2-dimethyl-[1,3]dioxin-4-ones (formula VII), which serve as building blocks in the synthesis of the target compounds (Synthetic Scheme H):

General Procedure N

Preparation of 6-aryl-2,2-dimethyl-[1,3]dioxin-4-ones

Method a)

The 6-aryl-2,2-dimethyl-[1,3]dioxin-4-ones were prepared from 3-aryl-3-oxo-propionic acids and catalytic amount of conc. H_2SO_4 or trifluoroacetic acid (TFA) in isopropenyl acetate at 23° C. according to *Chem. Pharm. Bull.* 1983, 31, 1896. The final products were purified by silica gel column chromatography with hexane/EtOAc.

Method b)

The 6-aryl-2,2-dimethyl-[1,3]dioxin-4-ones were prepared from the tert.-butyl 3-aryl-3-oxo-propionates by treatment with trifluoroacetic anhydride (TFAA) in a mixture of TFA and acetone at 23° C. according to *Tetrahedron Lett.* 1998, 39, 2253. The final products were if necessary purified by silica gel column chromatography with hexane/EtOAc.

Example N1

2,2-Dimethyl-6-thiophen-2-yl-[1,3]dioxin-4-one

The 3-oxo-3-thiophen-2-yl-propionic acid was prepared from thiophene-2-carbonyl chloride (5.3 mL, 50 mmol) and bis(trimethylsilyl)malonate (25.6 mL, 100 mmol) with n-BuLi (1.6M in hexane, 62.5 mL) in ether at -60° C. to 0° C. according to the general procedure M (method c2). The crude material (7.88 g) was transformed into the title compound by stirring in isopropenyl acetate and TFA according to the general procedure N (method a). Obtained as a yellow solid (4.09 g).

MS (EI) 210 (M^+); mp 42° C. (dec.).

Example N2

6-(3-Chloro-thiophen-2-yl)-2,2-dimethyl-[1,3] dioxin-4-one

The 3-(3-chloro-thiophen-2-yl)-3-oxo-propionic acid was prepared from 3-chloro-thiophene-2-carbonyl chloride (7.82 g, 43.2 mmol) and bis(trimethylsilyl)malonate (11.6 mL, 45.4 mmol) with Et_3N (12.65 mL, 90.7 mmol) and LiBr (3.53 g, 47.5 mmol) in CH_3CN at 0° C. according to general procedure M (method c1). The crude material (5.69 g) was transformed into the title compound by stirring in isopropenyl acetate and conc. H_2SO_4 according to general procedure N (method a). Obtained as an orange solid (2.3 g).

MS (EI) 244 (M^+) and 246 [$(M+2)^+$]; mp 88–89° C. (dec.).

Example N3

6-(3-Cyano-thiophen-2-yl)-2,2-dimethyl-[1,3]dioxin-4-one

The 3-(3-cyano-thiophen-2-yl)-3-oxo-propionic acid was prepared from 3-cyano-thiophene-2-carbonyl chloride (24.33 g, 140.6 mmol) and bis(trimethylsilyl)malonate (38.0 mL, 147.7 mmol) with Et_3N (41 mL, 295.4 mmol) and LiBr (13.5 g, 154.7 mmol) in CH_3CN at 0° C. according to general procedure M (method c1). The crude material (24.8 g) was transformed into the title compound by stirring in

isopropenyl acetate and conc. H_2SO_4 according to general procedure N (method a). Obtained as an orange solid (5.6 g).

MS (EI) 235 (M^+); mp 116–120° C. (dec.).

Example N4

3-(2,2-Dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile

The 3-(3-cyano-phenyl)-3-oxo-propionic acid was prepared from 3-cyanobenzoyl chloride (828 mg, 5 mmol) and bis(trimethylsilyl)malonate (2.56 mL, 10 mmol) with n-BuLi (1.6M in hexane, 6.25 mL) in ether at −60° C. to 0° C. according to general procedure M (method c2). The crude material (1.04 g) was transformed into the title compound by stirring in isopropenyl acetate and TFA according to general procedure N (method a). Obtained as a light yellow solid (0.8 g).

MS (EI) 229 (M^+); mp 138° C. (dec.).

Example N5

2,2-Dimethyl-6-(3-trifluoromethyl-phenyl)-[1,3]dioxin-4-one

The 3-oxo-3-(3-trifluoromethyl-phenyl)-propionic acid was prepared from 3-trifluoromethylbenzoyl chloride (10 mL, 67.6 mmol) and bis(trimethylsilyl)malonate (18.2 mL, 71 mmol) with Et_3N (20 mL, 142 mmol) and LiBr (6.46 g, 74.4 mmol) in CH_3CN at 0° C. according to general procedure M (method c1). The crude material (7.0 g of the obtained 15.4 g) was transformed into the title compound by stirring in isopropenyl acetate and conc. H_2SO_4 according to general procedure N (method a). Obtained as a light yellow solid (5.3 g).

MS (EI) 272 (M^+); mp 77–78° C. (dec.).

Example N6

6-(3-Chloro-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one

The 3-(3-chloro-phenyl)-3-oxo-propionic acid was prepared from 3-chlorobenzoyl chloride (11 mL, 85.7 mmol) and bis(trimethylsilyl)malonate (23.0 mL, 90.0 mmol) with Et_3N (25 mL, 180 mmol) and LiBr (8.19 g, 94.3 mmol) in CH_3CN at 0° C. according to general procedure M (method c1). The crude material (17.1 g) was transformed into the title compound by stirring in isopropenyl acetate and conc. H_2SO_4 according to general procedure N (method a). Obtained as a yellow-brown solid (8.0 g).

MS (EI) 238 (M^+) and 240 [$(M+2)^+$]; mp 87–88° C. (dec.).

Example N7

6-(3-Iodo-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one

The 3-(3-iodo-phenyl)-3-oxo-propionic acid was prepared from 3-iodobenzoyl chloride (21.0 g, 78.8 mmol) and bis(trimethylsilyl)malonate (21.0 mL, 82.8 mmol) with Et_3N (23 mL, 165.5 mmol) and LiBr (7.54 g, 86.7 mmol) in CH_3CN at 0° C. according to general procedure M (method c1). The crude material (21.9 g) was transformed into the title compound by stirring in isopropenyl acetate and conc. H_2SO_4 according to general procedure N (method a). Obtained as a yellow solid (9.6 g).

MS (EI) 330 (M^+); mp 79–80° C. (dec.).

Example N8

2,2-Dimethyl-6-(3-trifluoromethoxy-phenyl)-[1,3]dioxin-4-one

The 3-oxo-3-(3-trifluoromethoxy-phenyl)-propionic acid was prepared from 3-trifluoromethoxybenzoyl chloride and bis(trimethylsilyl)malonate with Et_3N and LiBr in CH_3CN at 0° C. according to general procedure M (method c1). The crude material was transformed into the title compound by stirring in isopropenyl acetate and conc. H_2SO_4 according to general procedure N (method a). Obtained as an orange solid (2.27 g).

MS (EI) 288 (M^+); mp 49–54° C. (dec.).

Example N9

2,2-Dimethyl-6-pyridin-4-yl-[1,3]dioxin-4-one

Prepared from 3-oxo-3-pyridin-4-yl-propionic acid [prepared from 4-acetylpyridine, magnesium methylcarbonate and CO_2 in DMF at 120° C. according to *Journal of Antibiotics* 1978, 31, 1245] by treatment with acetone, TFA and TFAA according to general procedure N (method b). Obtained as a white solid (1.3 g).

MS (EI) 205 (M^+)

Example N10

6-(3-Imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one

The 3-(3-imidazol-1-yl-phenyl)-3-oxo-propionic acid was prepared from 3-(1H-imidazol-1-yl)benzoyl chloride hydrochloride [prepared by treatment of 3-(1H-imidazol-1-yl)benzoic acid (*J. Med. Chem.* 1987, 30, 1342; CAS-No. [108035-47-8] with $SOCl_2$) and bis(trimethylsilyl)malonate with Et_3N and LiBr in CH_3CN at 0° C. according to general procedure M (method c1). The crude material was transformed into the title compound by stirring in isopropenyl acetate and conc. H_2SO_4 according to general procedure N (method a). Obtained as an orange semisolid (617 mg).

MS (EI) 270 (M^+).

Example N11

2,2-Dimethyl-6-(3-methoxy-phenyl)-[1,3]dioxin-4-one

The 3-(3-methoxy-phenyl)-3-oxo-propionic acid was prepared from 3-methoxybenzoyl chloride (10.3 g, 60.4 mmol) and bis(trimethylsilyl)malonate (16.2 mL, 63.4 mmol) with Et_3N (17.7 mL, 127 mmol) and LiBr (5.77 g, 66.4 mmol) in CH_3CN at 0° C. according to general procedure M (method c1). The crude material (6.38 g) was transformed into the title compound by stirring in isopropenyl acetate and conc. H_2SO_4 according to general procedure N (method a). Obtained as a yellow oil (640 mg).

MS (ISP) 235 [$(M+H)^+$] and 252 [$(M+NH_4)^+$].

Example N12

2,2-Dimethyl-6-(3-nitro-phenyl)-[1,3]dioxin-4-one

The 3-(3-nitro-phenyl)-3-oxo-propionic acid tert.-butyl ester was prepared from 3-nitrobenzoyl chloride (2.71 g, 14.6 mmol) and tert.-butyl malonate potassium salt (6.0 g, 30.0 mmol) with Et_3N (4.5 mL, 32.2 mmol) and $MgCl_2$ (3.48 g, 36.52 mmol) in CH_3CN according to general procedure M (method a). The crude material (3.88 g) was transformed into the title compound by stirring in TFA/acetone with TFAA according to general procedure N (method b). Obtained as a yellow solid (2.76 g).

MS (EI) 249 (M^+); mp 110–117° C.

Example N13

2,2-Dimethyl-6-(3-[1,2,4]triazol-1-yl-phenyl)-[1,3]dioxin-4-one

The 3-oxo-3-(3-[1,2,4]triazol-1-yl-phenyl)-propionic acid tert.-butyl ester [RO-69-3506/000] was prepared from 3-[1,

2,4]triazol-1-yl-benzoic acid methyl ester [CAS-No. 167626-27-9] by treatment with lithium tert.-butyl acetate according to general procedure M (method b). Prepared from (Example M6) by stirring in TFA/acetone with TFAA according to general procedure N (method b). Obtained as a yellow solid (539 mg).

MS (EI) 271 (M^+).

Example N14

6-(2-Imidazol-1-yl-pyridin-4-yl)-2,2-dimethyl-[1,3]dioxin-4-one

Prepared from 3-(2-imidazol-1-yl-pyridin-4-yl)-3-oxo-propionic acid tert.-butyl ester (Example M5) by stirring in TFA/acetone with TFAA according to general procedure N (method b). Obtained as a brown solid (10.8 g).

MS (EI) 271 (M^+); mp 151° C. (dec.).

Example N15

2,2-Dimethyl-6-[3-(2-methyl-imidazol-1-yl)-phenyl]-[1,3]dioxin-4-one

Prepared from 3-[3-(2-methyl-imidazol-1-yl)-phenyl]-3-oxo-propionic acid tert.-butyl ester (Example M8) by stirring in TFA/acetone with TFAA according to general procedure N (method b). Obtained as a beige solid (2.13 g).

MS (EI) 284 (M^+); mp 122° C.

Example N16

4-(2,2-Dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-pyridine-2-carbonitrile

Prepared from 3-(2-cyano-pyridin-4-yl)-3-oxo-propionic acid tert.-butyl ester (Example M10) by stirring in TFA/acetone with TFAA according to general procedure N (method b). Obtained as a brown solid (3.30 g).

MS (EI) 230 (M^+); mp 132° C. (dec.).

The following examples relate to the preparation of the 4,8-diaryl-1,3-dihydro-benzo[b][1,4]diazepin-2-ones, respectively the 4-aryl-8-arylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-ones and the 8-aroyl-4-aryl-1,3-dihydro-benzo[b][1,4]diazepin-2-ones in regioisomerically pure fashion (Synthetic Scheme A):

General Procedure O

Preparation of {2-[3-aryl-3-oxo-propionylamino]-4-aryl-phenyl}-carbamic acid tert.-butyl ester by reaction of (2-amino-4-aryl-phenyl)-carbamic acid tert.-butyl esters with ethyl 3-aryl-3-oxo-propionates or 6-aryl-2,2-dimethyl-[1,3]dioxin-4-ones

also 3-aryl-N-(2-nitro-4-aryl-phenyl)-3-oxo-propionamides by reaction of 2-nitro-4-aryl-phenylamines with 6-aryl-2,2-dimethyl-[1,3]dioxin-4-ones

A mixture of the (2-amino-4-aryl-phenyl)-carbamic acid tert.-butyl ester or 2-nitro-4-aryl-phenylamine (1.0 mmol) and excess (1.2–1.5 mmol) of the ethyl 3-aryl-3-oxo-propionate [prepared from the aryl acid chloride and ethyl malonate potassium salt with Et_3N and $MgCl_2$ in CH_3CN at 23° C. according to *Synthesis* 1993, 290] or 6-aryl-2,2-dimethyl-[1,3]dioxin-4-one was refluxed in toluene (8 mL) until tlc indicated complete consumption of the amine. The solution was allowed to cool to 23° C., whereupon the product generally crystallized (in cases where crystallization failed to appear it was induced by addition of hexane). The solid was filtered off, washed with ether or mixtures of ether/hexane and dried in vacuum to give the {2-[3-aryl-3-oxo-propionylamino]-4-aryl-phenyl}-carbamic acid tert.-butyl esters or 3-aryl-N-(2-nitro-4-aryl-phenyl)-3-oxo-propionamides, which was used directly in the following step or —if necessary —was purified by recrystallization or by silica gel column chromatography.

Example O1

{2-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-4-iodo-5-thiomorpholin-4-yl-phenyl}-carbamic acid tert.-butyl ester

Prepared from (2-amino-4-iodo-5-thiomorpholin-4-yl-phenyl)-carbamic acid tert.-butyl ester (Example L1) (653 mg, 1.5 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (690 mg, 2.25 mmol) according to the general procedure O. Obtained as a yellow solid (629 mg).

MS (ISP) 607 [$(M+H)^+$].

Example O2

{2-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-4-iodo-5-morpholin-4-yl-phenyl}-carbamic acid tert.-butyl ester

Prepared from (2-amino-4-iodo-5-morpholin-4-yl-phenyl)-carbamic acid tert.-butyl ester (Example L2) (690 mg, 1.65 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (566 mg, 2.47 mmol) according to the general procedure O. Obtained as an orange solid (523 mg).

MS (ISP) 591 [$(M+H)^+$] and 613 [$(M+Na)^+$].

Example O3

{2-Chloro-5-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-chloro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L6) (652 mg, 2.05 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (564 mg, 2.46 mmol) according to the general procedure O. Obtained as an off-white solid (725 mg).

MS (ISN) 488 [$(M-H)^-$].

Example O4

[2-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-5-(2-dimethylamino-ethylsulfanyl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [2-amino-5-(2-dimethylamino-ethylsulfanyl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example L8) (206 mg, 0.5 mmol) and 3-(2,2-dimethyl-6-oxo-6 H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (138 mg, 0.6 mmol) according to the general procedure O. Obtained as a yellow solid (210 mg).

MS (ISP) 583 [$(M+H)^+$]; mp 88° C.

Example O5

{5-tert.-Butoxycarbonylamino-4-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2-phenylethynyl-phenylsulfanyl}-acetic acid methyl ester

Prepared from (4-amino-5-tert.-butoxycarbonylamino-2-phenylethynyl-phenylsulfanyl)-acetic acid methyl ester

(Example L9) (534 mg, 1.3 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (358 mg, 1.56 mmol) according to the general procedure O. Obtained as a yellow foam (457 mg).

MS (ISP) 584 [(M+H)$^+$], 601 [(M+NH$_4$)$^+$] and 605 [(M+Na)$^+$]; mp 69–73° C.

Example O6

{5-tert.-Butoxycarbonylamino-4-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2-phenylethynyl-phenyl}-acetic acid methyl ester

Prepared from (4-amino-5-tert.-butoxycarbonylamino-2-phenylethynyl-phenyl)-acetic acid methyl ester (Example L10) (721 mg, 2.0 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (550 mg, 2.4 mmol) according to the general procedure O. Obtained as a light yellow solid (886 mg).

MS (ISP) 552 [(M+H)$^+$] and 569 [(M+NH$_4$)$^+$]; mp 138° C.

Example O7

[2-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-5-(2-methoxy-ethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [2-amino-5-(2-methoxy-ethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example L11) (415 mg, 1.09 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (373 mg, 1.63 mmol) according to the general procedure O. Obtained as a light yellow solid (137 mg).

MS (ISP) 554 [(M+H)$^+$], 571 [(M+NH$_4$)$^+$] and 576 [(M+Na)$^+$]; mp 175–176° C.

Example O8

{2-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-5-methoxy-4-phenylethynyl-phenyl}-carbamic acid tert.-butyl ester

Prepared from (2-amino-5-methoxy-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester (Example L12) (338 mg, 1.0 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (252 mg, 1.1 mmol) according to the general procedure O. Obtained as a yellow solid (388 mg).

MS (ISP) 510 [(M+H)$^+$], 527 [(M+NH$_4$)$^+$] and 532 [(M+Na)$^+$]; mp 169° C.

Example O9

5-tert.-Butoxycarbonylamino-4-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2-phenylethynyl-benzoic acid methyl ester

Prepared from 4-amino-5-tert.-butoxycarbonylamino-2-phenylethynyl-benzoic acid methyl ester (Example L14) (396 mg, 1.08 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (273 mg, 1.19 mmol) according to the general procedure O. Obtained as an off-white solid (540 mg).

MS (ISP) 538 [(M+H)$^+$], 555 [(M+NH$_4$)$^+$] and 560 [(M+Na)$^+$]; mp 158° C. (dec.).

Example O10

{2-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-5-morpholin-4-yl-4-phenylethynyl-phenyl}-carbamic acid tert.-butyl ester

Prepared from (2-amino-5-morpholin-4-yl-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester (Example L12) (483 mg, 1.23 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (422 mg, 1.84 mmol) according to the general procedure O. Obtained as an amorphous orange material (375 mg).

MS (ISP) 565 [(M+H)$^+$] and 587 [(M+Na)$^+$].

Example O11

[2-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-5-(2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [2-amino-5-(2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example L15) (514 mg, 1.0 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (344 mg, 1.5 mmol) according to the general procedure O. Obtained as a light yellow solid (353 mg).

MS (ISP) 686 [(M+H)$^+$] and 703 [(M+NH$_4$)$^+$]; mp 135–136° C.

Example O12

{5-tert.-Butoxycarbonylamino-4-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2-phenylethynyl-phenoxy}-acetic acid tert.-butyl ester

Prepared from (4-amino-5-tert.-butoxycarbonylamino-2-phenylethynyl-phenoxy)-acetic acid tert.-butyl ester (Example L16) (877 mg, 2.0 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (504 mg, 2.2 mmol) according to the general procedure O. Obtained as a yellow solid (723 mg).

MS (ISP) 610 [(M+H)$^+$], 627 [(M+NH$_4$)$^+$] and 632 [(M+Na)$^+$]; mp 95° C.

Example O13

{5-Cyanomethyl-2-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-4-phenylethynyl-phenyl}-carbamic acid tert.-butyl ester

Prepared from (2-amino-5-cyanomethyl-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester (Example L17) (298 mg, 0.86 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (218 mg, 0.95 mmol) according to the general procedure O. Obtained as a yellow solid (299 mg).

MS (ISP) 519 [(M+H)$^+$], 536 [(M+NH$_4$)$^+$] and 541 [(M+Na)$^+$]; mp 98° C.

Example O14

[2-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-5-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [2-amino-5-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example L18) (393 mg, 0.87 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (301 mg, 1.31 mmol) according to the general procedure O. Obtained as an amorphous orange material (268 mg).

MS (ISP) 621 [(M+H)$^+$].

Example O15

[2-[3-(3-Iodo-phenyl)-3-oxo-propionylamino]-5-(2-methoxy-ethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [2-amino-5-(2-methoxy-ethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

(Example L11) (840 mg, 2.2 mmol) and 6-(3-iodo-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N7) (780 mg, 2.36 mmol) according to the general procedure O. Obtained as a white solid (1.3 g).

MS (ISP) 655 [(M+H)$^+$], 672 [(M+NH4)$^+$], 677 [(M+Na)$^+$] and 693 [(M+K)$^+$]; mp 172° C.

Example O16

[2-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-5-(2,2-dimethyl-[1,3]dioxolan-4-ylmethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [2-amino-5-(2,2-dimethyl-[1,3]dioxolan-4-ylmethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example L20) (439 mg, 1.0 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (345 mg, 1.5 mmol) according to the general procedure O. Obtained as a yellow solid (275 mg).

MS (ISP) 610 [(M+H)$^+$], 627 [(M+NH$_4$)$^+$] and 632 [(M+Na)$^+$]; mp 132–134° C.

Example O17

5-tert.-Butoxycarbonylamino-4-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2-iodo-benzoic acid methyl ester

Prepared from 4-amino-5-tert.-butoxycarbonylamino-2-iodo-benzoic acid methyl ester (Example L21) (395 mg, 1.0 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (254 mg, 1.1 mmol) according to the general procedure O. Obtained as an apricot solid (435 mg).

MS (ISP) 564 [(M+H)$^+$], 581 [(M+NH$_4$)$^+$] and 586 [(M+Na)$^+$]; mp 162–166° C.

Example O18

[[2-[3-(3-Imidazol-1-yl-phenyl)-3-oxo-propionylamino]-5-(2-methoxy-ethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [2-amino-5-(2-methoxy-ethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example L11) (191 mg, 0.5 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (135 mg, 0.5 mmol) according to the general procedure O. Obtained as a light brown waxy solid (206 mg).

MS (ISN) 593 [(M–H)$^-$]; mp 122–129° C.

Example O19

[RS]-[2-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-5-(2-oxo-[1,3]dioxolan-4-ylmethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [RS]-[2-amino-5-(2-oxo-[1,3]dioxolan-4-ylmethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example L22) (346 mg, 0.82 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (206 mg, 0.9 mmol) according to the general procedure K. Obtained as a light yellow solid (433 mg).

MS (ISP) 594 [(M–H)$^+$]; mp 181° C.

Example O20

{2-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-5-ethoxymethyl-4-phenylethynyl-phenyl}-carbamic acid tert.-butyl ester

Prepared from (2-amino-5-ethoxymethyl-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester (Example L23) (130 mg, 0.35 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (80 mg, 0.35 mmol) according to the general procedure K. Obtained as an amorphous yellow substance (148 mg).

MS (ISP) 538 [(M–H)$^+$].

Example O21

2,2-Dimethyl-propionic acid 5-tert.-butoxycarbonylamino-4-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2-phenylethynyl-benzyl ester

Prepared from 2,2-dimethyl-propionic acid 4-amino-5-tert.-butoxycarbonylamino-2-phenylethynyl-benzyl ester (Example L24) (155 mg, 0.37 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (94 mg, 0.41 mmol) according to the general procedure K. Obtained as an amorphous light orange substance (184 mg).

MS (ISP) 611 [(M+NH$_4$)$^+$].

Example O22

(RS)-[2-[3-(3-Imidazol-1-yl-phenyl)-3-oxo-propionylamino]-4-phenylethynyl-5-(tetrahydro-pyran-2-yloxymethyl)-phenyl]-carbamic acid tert.-butyl ester

Prepared from (RS)-[2-amino-4-phenylethynyl-5-(tetrahydro-pyran-2-yloxymethyl)-phenyl]-carbamic acid tert.-butyl ester (Example L25) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) according to the general procedure O. Obtained as an amorphous yellow substance (267 mg).

MS (ISP) 635 [(M+H)$^+$].

Example O23

[2-[3-(3-Imidazol-1-yl-phenyl)-3-oxo-propionylamino]-5-(4-methoxy-piperidin-1-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [2-amino-5-(4-methoxy-piperidin-1-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example L26) (236 mg, 0.56 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (151 mg, 0.56 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (253 mg).

MS (ISP) 634 [(M+H)$^+$].

Example O24

[2-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-5-(4-methoxy-piperidin-1-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester

Prepared from [2-amino-5-(4-methoxy-piperidin-1-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example L26) (224 mg, 0.53 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (125 mg, 0.53 mmol) according to the general procedure O. Obtained as a yellow foam (274 mg).

MS (ISN) 591 [(M–H)$^-$]; mp 97–100° C.

Example O25

{5-Cyanomethoxy-2-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-4-phenylethynyl-phenyl}-carbamic acid tert.-butyl ester

Prepared from (2-amino-5-cyanomethoxy-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester

(Example L27) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) according to the general procedure O. Obtained as an amorphous yellow substance (169 mg).

MS (ISP) 576 [(M+H)$^+$].

Example O26

{5-Cyanomethoxy-2-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-4-phenylethynyl-phenyl}-carbamic acid tert.-butyl ester

Prepared from (2-amino-5-cyanomethoxy-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester (Example L27) (80 mg, 0.22 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl) -benzonitrile (Example N4) (55 mg, 0.24 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (96 mg).

MS (ISN) 533 [(M−H)$^-$].

Example O27

(RS)-[4-(4-Fluoro-phenylethynyl)-2-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-5-(tetrahydro-pyran-2-yloxymethyl)-phenyl]-carbamic acid tert.-butyl ester

Prepared from (RS)-2-amino-4-(4-fluoro-phenylethynyl)-5-(tetrahydro-pyran-2-yloxymethyl)-phenyl]-carbamic acid tert.-butyl ester (Example L28) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) according to the general procedure O. Obtained as an amorphous yellow substance (990 mg).

MS (ISN) 651 [(M−H)$^-$].

Example O28

(RS)-(4-(4-Fluoro-phenylethynyl)-2-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-5-{methyl-[2-(tetrahydro-pyran-2-yloxy)-ethyl]-amino}-phenyl)-carbamic acid tert.-butyl ester

Prepared from (RS)-(2-amino-4-(4-fluoro-phenylethynyl)-5-{methyl-[2-(tetrahydro-pyran-2-yloxy)-ethyl]-amino}-phenyl)-carbamic acid tert.-butyl ester (Example L29) (242 mg, 0.5 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (135 mg, 0.5 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (198 mg).

MS (ISN) 694 [(M−H)$^-$].

Example O29

{5-(4,4-Diethoxy-piperidin-1-yl)-2-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-4-phenylethynyl-phenyl}-carbamic acid tert.-butyl ester

Prepared from [2-amino-5-(4,4-diethoxy-piperidin-1-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example L18) (321 mg, 0.67 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (240 mg, 0.89 mmol) according to the general procedure O. Obtained as an orange foam (295 mg).

MS (ISP) 692 [(M+H)$^+$].

Example O30

(RS)-{2-[3-(3-Imidazol-1-yl-phenyl)-3-oxo-propionylamino]-4-phenylethynyl-5-[2-(tetrahydro-pyran-2-yloxy)-ethoxy]-phenyl}-carbamic acid tert.-butyl ester

Prepared from (RS)-{2-amino-4-phenylethynyl-5-[2-(tetrahydro-pyran-2-yloxy)-ethoxy]-phenyl}-carbamic acid tert.-butyl ester (Example L30) (346 mg, 0.76 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (300 mg, 1.11 mmol) according to the general procedure O. Obtained as a yellow foam (196 mg).

MS (ISP) 665 [(M+H)$^+$].

Example O31

(RS)-{4'-Fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-[4-(tetrahydro-pyran-2-yloxy)-piperidin-1-yl]-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (RS)-[2-amino-4-(4-fluoro-phenylethynyl)-5-(tetrahydro-pyran-2-yloxymethyl)-phenyl]-carbamic acid tert.-butyl ester (Example L28) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) according to the general procedure O. Obtained as a yellow solid (282 mg).

MS (ISP) 698 [(M+H)$^+$]; mp 129–132° C.

Example O32

{5-(2-tert.-Butoxy-ethoxy)-4-(4-fluoro-phenylethynyl)-2-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-phenyl}-carbamic acid tert.-butyl ester

Prepared from [2-amino-5-(2-tert.-butoxy-ethoxy)-4-(4-fluoro-phenylethynyl)-phenyl]-carbamic acid tert.-butyl ester (Example L32) (560 mg, 1.27 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10O) (413 mg, 1.53 mmol) according to the general procedure O. Obtained as a yellow solid (507 mg).

MS (ISP) 655 [(M+H)$^+$]; mp 62–65° C.

Example O33

(RS)-(4'-Fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-{4-[2-(tetrahydro-pyran-2-yloxy)-ethoxy]-piperidin-1-yl}-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (RS)-(5-amino-4'-fluoro-2-{4-[2-(tetrahydro-pyran-2-yloxy)-ethoxy]-piperidin-1-yl}-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L33) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) according to the general procedure O. Obtained as a yellow solid (473 mg).

MS (ISP) 742 [(M+H)$^+$]; mp 57–58° C.

Example O34

(RS)-[4'-Fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-(tetrahydro-pyran-2-yloxymethyl)-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from (RS)-[5-amino-4'-fluoro-2-(tetrahydro-pyran-2-yloxymethyl)-biphenyl-4-yl]-carbamic acid tert-butyl ester (Example L34) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) according to the general procedure O. Obtained as a light yellow solid (330 mg).

Example O35

{2-Cyanomethoxy-4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-cyanomethoxy-4'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example

L35) (190 mg, 0.53 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (164 mg, 0.61 mmol) according to the general procedure O. Obtained as a yellow gum (90 mg).

MS (ISP) 570 [(M+H)$^+$].

Example O36

{2-Dimethylaminomethyl-4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-dimethylaminomethyl-4'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L36) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) according to the general procedure O. Obtained as a light yellow solid (329 mg).

Example O37

{2-(2,2-Dimethyl-tetrahydro-[1,3]dioxolo[4,5-c]pyrrol-5-yl)-4'-fluoro-5-[3-(3-imidazol-1yl-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from [5-amino-2-(2,2-dimethyl-tetrahydro-[1,3]dioxolo [4,5-c]pyrrol-5-yl)-4'-fluoro-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example L37) (465 mg, 1.05 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (314 mg, 1.16 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (540 mg).

MS (ISN) 654 [(M−H)$^-$].

Example O38

{4'-Fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-methoxy-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-4'-fluoro-2-methoxy-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L38) (332 mg, 1.0 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (270 mg, 1.0 mmol) according to the general procedure O. Obtained as an amorphous brown substance (328 mg).

MS (ISN) 543 [(M−H)$^-$].

Example O39

{2-(1,4-Dioxa-8-aza-spiro[4.5]dec-8-yl)-4'-fluoro-5-[3-(3-Imidazol-1yl-phenyl)-3oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from [5-amino-2-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-4'-fluoro-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example L39) (444 mg, 1.0 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (321 mg, 1.19 mmol) according to the general procedure O. Obtained as a brown solid (457 mg).

MS (ISN) 654 [(M−H)$^-$]; mp 110–115° C. (dec.).

Example O40

{4'-Fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-methyl-biphenyl-4yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-4'-fluoro-2-methyl-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L40) (316 mg, 1.0 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (297 mg, 1.1 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (361 mg).

MS (ISP) 529 [(M+H)$^+$].

Example O41

{4-tert.-Butoxycarbonylamino-4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-2-yloxy}-acetic acid tert.-butyl ester

Prepared from (5-amino-4-tert.-butoxycarbonylamino-4'-fluoro-biphenyl-2-yloxy)-acetic acid tert.-butyl ester (Example L41) (1.4 g, 3.24 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (885 mg, 3.27 mmol) according to the general procedure O. Obtained as a light brown solid (759 mg).

MS (ISP) 645 [(M+H)$^+$]; mp 82–85° C.

Example O42

{2-Chloro-4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-4yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-chloro-4'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L42) (168 mg, 0.5 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (252 mg, 0.93 mmol) according to the general procedure O. Obtained as a light yellow solid (156 mg).

MS (ISN) 547 [(M−H)$^-$].

Example O43

[4'-Fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-(2-methoxy-ethoxy)-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [5-amino-4'-fluoro-2-(2-methoxy-ethoxy)-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example L43) (188 mg, 0.5 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (260 mg, 0.96 mmol) according to the general procedure O. Obtained as an orange solid (218 mg).

MS ([SP) 589 [(M+H)$^+$]; mp 61–63° C.

Example O44

{2-(2-tert.-Butoxy-ethoxy)-4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from [5-amino-2-(2-tert.-butoxy-ethoxy)-4'-fluoro-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example L44) (209 mg, 0.5 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (135 mg, 0.5 mmol) according to the general procedure O. Obtained as a beige solid (194 mg).

MS (ISP) 631 [(M+H)$^+$]; mp 101° C.

Example O45

[4'-Fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-(2-oxo-oxazolidin-3yl)-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [5-amino-4'-fluoro-2-(2-oxo-oxazolidin-3-yl)-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example

L45) (130 mg, 0.34 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (92 mg, 0.34 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (86 mg).

MS (ISP) 600 [(M+H)$^+$].

Example O46

{4'-Fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-methoxy-2'-methyl-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-4'-fluoro-2-methoxy-2'-methyl-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L46) (173 mg, 0.5 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (199 mg, 1.47 mmol) according to the general procedure O. Obtained as an orange solid (182 mg).

MS (ISP) 559 [(M+H)$^+$]; mp 99–102° C.

Example O47

{2-tert.-Butoxy-4'-fluoro-5-(3-(3-imidazol-1-yl-phenyl[-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-tert.-butoxy-4'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L47) (187 mg, 0.5 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (135 mg, 0.5 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (237 mg).

MS (ISN) 585 [(M–H)$^-$].

Example O48

{2-tert.-Butoxy-2'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-tert.-butoxy-2'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L48) (187 mg,0.5 mmol) and 6-(3-imidazol-1-yl-phenyl)-2, 2-dimethyl-[1,3]dioxin-4-one (Example N10) (135 mg, 0.5 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (234 mg).

MS (ISN) 585 [(M–H)$^-$].

Example O49

(RS)-{4'-Fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-[(R)-3-(tetrahydro-pyran-2-yloxy)-pyrrolidin-1-yl]-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (RS)-{5-amino-4'-fluoro-2-[(R)-3-(tetrahydro-pyran-2-yloxy)-pyrrolidin-1-yl]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example L49) (236 mg, 0.5 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1, 3]dioxin-4-one (Example N 10) (200 mg, 0.74 mmol) according to the general procedure O. Obtained as an orange solid (188 mg).

MS (ISP) 684 [(M+H)$^+$]; mp 99–103° C.

Example O50

{2'-Fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-methoxy-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2'-fluoro-2-methoxy-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L50) (159 mg, 0.5 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (135 mg, 0.5 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (199 mg).

MS (ISN) 543 [(M–H)$^-$].

Example O51

{2-tert.-Butoxy-5-[3-(3-cyano-phenyl)-3-oxo-propionylamino]4'-fluoro-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-tert.-butoxy-4'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L47) (187 mg, 0.5 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (126 mg, 0.55 mmol) according to the general procedure O. Obtained as an amorphous light pink substance (196 mg).

MS (ISP) 546 [(M+H)$^+$].

Example O52

{2-tert.-Butoxy-5-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2'-fluoro-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-tert.-butoxy-2'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L48) (187 mg, 0.5 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (126 mg, 0.55 mmol) according to the general procedure O. Obtained as an amorphous light pink substance (197 mg).

MS (ISP) 546 [(M+H)$^+$].

Example O53

{5-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-2'-fluoro-2-methoxy-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2'-fluoro-2-methoxy-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L50) (112 mg, 0.35 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (80 mg, 0.35 mmol) according to the general procedure O. Obtained as a yellow foam (131 mg).

MS (ISN) 502 [(M–H)$^-$].

Example O54

(2'-Fluoro-2-methoxy-5-{3-[3-(2-methyl-imidazol-1-yl)-phenyl]-3-oxo-propionylamino}-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (5-amino-2'-fluoro-2-methoxy-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L50) (249 mg, 0.75 mmol) and 3-[3-(2-methyl-imidazol-1-yl)-phenyl]-3-oxo-propionic acid tert.-butyl ester (Example M8) (275 mg, 0.92 mmol) according to the general procedure O. Obtained as a yellow solid (312 mg).

MS (ISP) 559 [(M+H)$^+$]; mp 83–86° C.

Example O55

{5-[3-(5-Cyano-thiophen-2-yl)-3-oxo-propionylamino]-2'-fluoro-2-methoxy-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2'-fluoro-2-methoxy-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L50) (166

mg, 0.5 mmol) 3-(5-cyano-thiophen-2-yl)-3-oxo-propionic acid tert.-butyl ester (Example M17) (138 mg, 0.55 mmol) according to the general procedure O. Obtained as a light yellow solid (244 mg).

MS (ISP) 510 [(M+H)$^+$]; mp 200° C. (dec.).

Example O56

{2'-Fluoro-2-methoxy-5-[3-oxo-3-(3-[1,2,4]triazol-4-yl-phenyl)-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2'-fluoro-2-methoxy-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L50) (166 mg, 0.5 mmol) and 3-oxo-3-(3-[1,2,4]triazol-4-yl-phenyl)-propionic acid ethyl ester (Example M1) (260 mg, 1.0 mmol) according to the general procedure O. Obtained as a yellow gum (70 mg).

MS (ISP) 546 [(M+H)$^+$].

Example O57

{5-[3-(2-Cyano-pyridin-4-yl)-3-oxo-propionylamino]-2'-fluoro-2-methoxy-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2'-fluoro-2-methoxy-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L50) and 3-(2-cyano-pyridin-4-yl)-3-oxo-propionic acid tert.-butyl ester (Example M10) according to the general procedure O. Obtained as a light yellow solid (189 mg).

MS (ISP) 522 [(M+NH_4)$^+$].

Example O58

(2-tert.-Butoxy-4'-fluoro-5-{3-[3-(2-methyl-imidazol-1-yl)-phenyl]-3-oxo-propionylamino}-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-tert.-butoxy-4'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L47) (140 mg, 0.37 mmol) and 3-[3-(2-methyl-imidazol-1-yl)-phenyl]-3-oxo-propionic acid tert.-butyl ester (Example M8) (111 mg, 0.37 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (139 mg).

MS (ISP) 601 [(M+H)$^+$].

Example O59

{5-[3-(5-Cyano-2-fluoro-phenyl)-3-oxo-propionylamino]-2'-fluoro-2-methoxy-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2'-fluoro-2-methoxy-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L50) (166 mg, 0.5 mmol) and 3-(5-cyano-2-fluoro-phenyl)-3-oxo-propionic acid ethyl ester (Example M18) (141 mg, 0.6 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (165 mg).

MS (ISP) 522 [(M+H)$^+$].

Example O60

{2-tert.-Butoxy-5-[3-(2-cyano-pyridin-4-yl)-3-oxo-propionylamino]-4'-fluoro-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-tert.-butoxy-4'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L47) (140 mg, 0.37 mmol) and 3-(2-cyano-pyridin-4-yl)-3-oxo-propionic acid tert.-butyl ester (Example M10) (91 mg, 0.37 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (164 mg).

MS (ISP) 547 [(M+H)$^+$].

Example O61

{2-tert.-Butoxy-2'-fluoro-5-[3-oxo-3-(3-[1,2,3]triazol-1-yl-phenyl)-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-tert.-butoxy-2'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L48) (187 mg,0.5 mmol) and 3-oxo-3-(3-[1,2,3]triazol-1-yl-phenyl)-propionic acid ethyl ester (Example M2) (180 mg, 0.69 mmol) according to the general procedure O. Obtained as a light yellow solid (257 mg).

MS (ISP) 588 [(M+H)$^+$]; mp 47–50° C.

Example O62

[5-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-2-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-2'-fluoro-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [5-amino-2-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-2'-fluoro-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example L51) (222 mg, 0.5 mmol) and 3-(3-cyano-phenyl)-3-oxo-propionic acid tert.-butyl ester (Example M3) (182 mg, 0.8 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (258 mg).

MS (ISP) 615 [(M+H)$^+$].

Example O63

{2-(1,4-Dioxa-8-aza-spiro[4.5]dec-8-yl)-2'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from [5-amino-2-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-2'-fluoro-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example L51) (222 mg, 0.5 mmol) and 6-(3-imidazol-1-yl-phenyl)-2,2-dimethyl-[1,3]dioxin-4-one (Example N10) (135 mg, 0.5 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (294 mg).

MS (ISP) 656 [(M+H)$^+$].

Example O64

(2-tert.-Butoxy-2'-fluoro-5-{3-[3-(2-methyl-imidazol-1-yl)-phenyl]-3-oxo-propionylamino}-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-tert.-butoxy-2'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L48) (187 mg, 0.5 mmol) and 2,2-dimethyl-6-[3-(2-methyl-imidazol-1-yl)-phenyl]-[1,3]dioxin-4-one (Example N15) (142 mg, 0.5 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (234 mg).

MS (ISP) 601 [(M+H)$^+$].

Example O65

{2-tert.-Butoxy-5-[3-(2-cyano-pyridin-4-yl)-3-oxo-propionylamino]-2'-fluoro-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-tert.-butoxy-2'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example

L48) (187 mg, 0.5 mmol) and 4-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl) -pyridine-2-carbonitrile (Example N16) (115 mg, 0.5 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (216 mg).

MS (ISP) 547 [(M+H)$^+$].

Example O66

(2-tert.-Butoxy-2'-fluoro-5-{3-[3-(2-methylsulfanyl-imidazol-1-yl)-phenyl]-3-oxo-propionylamino}-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-tert.-butoxy-2'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L48) (187 mg, 0.5 mmol) and 3-[3-(2-methylsulfanyl-imidazol-1-yl)-phenyl]-3-oxo-propionic acid tert.-butyl ester (Example M13) (211 mg, 0.63 mmol) according to the general procedure O. Obtained as a light yellow solid (260 mg).

MS (ISN) 631 [(M–H)$^-$]; mp 59–62° C.

Example O67

{5-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-2', 5'-difluoro-2-methoxy-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2',5'-difluoro-2-methoxy-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L52) (175 mg, 0.5 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (115 mg, 0.5 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (136 mg).

MS (ISP) 522 [(M+H)$^+$].

Example O68

{2',5'-Difluoro-2-methoxy-5-[3-oxo-3-(3-[1,2,3]triazol-1-yl-phenyl)-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2',5'-difluoro-2-methoxy-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L52) (175 mg, 0.5 mmol) and 3-oxo-3-(3-[1,2,3]triazol-1-yl-phenyl)-propionic acid ethyl ester (Example M2) (130 mg, 0.5 mmol) according to the general procedure O. Obtained as an amorphous light yellow substance (185 mg).

MS (ISN) 562 [(M–H)$^-$].

Example O69

{2-tert.-Butoxy-5-[3-(3-cyano-thiophen-2-yl)-3-oxo-propionylamino]-2'-fluoro-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-tert.-butoxy-2'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L48) (187 mg, 0.5 mmol) and 6-(3-cyano-thiophen-2-yl)-2, 2-dimethyl-[1,3]dioxin-4-one (Example N3) (130 mg, 0.55 mmol) according to the general procedure O. Obtained as a yellow oil (278 mg).

MS (ISN) 550 [(M–H)$^-$].

Example O70

{2-tert.-Butoxy-5-[3-(5-cyano-thiophen-2-yl)-3-oxo-propionylamino]-2'-fluoro-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-tert.-butoxy-2'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L48) (187 mg, 0.5 mmol) 3-(5-cyano-thiophen-2-yl)-3-oxo-propionic acid tert.-butyl ester (Example M17) (138 mg, 0.55 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (268 mg).

MS (ISN) 550 [(M–H)$^-$].

Example O71

{2-tert.-Butoxy-4'-fluoro-5-[3-oxo-3-(3-[1,2,3]triazol-1-yl-phenyl)-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-tert.-butoxy-4'-fluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L47) (187 mg, 0.5 mmol) and 3-oxo-3-(3-[1,2,3]triazol-1-yl-phenyl)-propionic acid ethyl ester (Example M2) (156 mg, 0.6 mmol) according to the general procedure O. Obtained as a yellow gum (198 mg).

MS (ISP) 588 [(M+H)$^+$].

Example O72

[5-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-2'-fluoro-2-(2-methoxy-ethoxy)-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from [5-amino-2'-fluoro-2-(2-methoxy-ethoxy)-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example L53) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) according to the general procedure O. Obtained as a yellow solid (188 mg).

MS (ISP) 548 [(M+H)$^+$].

Example O73

(RS)-[5-[3-(3-Cyano-phenyl)-3-oxo-propionylamino]-2'-fluoro-2-(tetrahydro-pyran-2-yloxymethyl)-biphenyl-4-yl]-carbamic acid tert.-butyl ester

Prepared from (RS)-[5-amino-2'-fluoro-2-(tetrahydro-pyran-2-yloxymethyl)-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example L54) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) according to the general procedure O. Obtained as a yellow solid (155 mg).

MS (ISP) 548 [(M+NH_4)$^+$].

Example O74

(2'-Fluoro-2-(4-methoxy-benzyloxy)-5-{3-[3-(3-methyl-isoxazol-5-yl)-phenyl]3-oxo-propionylamino}-biphenyl-4-yl)-carbamic acid tert.-butyl ester

Prepared [5-amino-2'-fluoro-2-(4-methoxy-benzyloxy)-biphenyl-4-yl -carbamic acid tert.-butyl ester (Example L55) (438 mg, 1.0 mmol) and 3-[3-(3-methyl-isoxazol-5-yl)-phenyl]-3-oxo-propionic acid tert.-butyl ester (Example M14) (301 mg, 1.0 mmol) according to the general procedure O. Obtained as an amorphous light yellow substance (561 mg).

MS (ISP) 666 [(M+H)$^+$].

Example O75

{2-tert.-Butoxy-5-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2',5'-difluoro-biphenyl-4-yl}-carbamic acid tert.-butyl ester

Prepared from (5-amino-2-tert.-butoxy-2',5'-difluoro-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example

L56) (196 mg, 0.5 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (115 mg, 0.5 mmol) according to the general procedure O. Obtained as an amorphous beige substance (155 mg).

MS (ISP) 564 [(M+H)$^+$].

Example O76

{5-tert.-Butoxy-4-(4-fluoro-phenylethynyl)-2-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-phenyl}-carbamic acid tert.-butyl ester

Prepared from [2-amino-5-tert.-butoxy-4-(4-fluoro-phenylethynyl)-phenyl]-carbamic acid tert.-butyl ester (Example L57) (160 mg, 0.4 mmol) and 3-(3-imidazol-1-yl-phenyl)-3-oxo-propionic acid tert.-butyl ester (Example M4) (115 mg, 0.4 mmol) according to the general procedure O. Obtained as an amorphous yellow substance (140 mg).

MS (ISP) 611 [(M+H)$^+$].

Example O77

{5-tert.-Butoxy-4-(4-fluoro-phenylethynyl)-2-[3-oxo-3-(3-[1,2,3]triazol-1-yl-phenyl)-propionylamino]-phenyl}-carbamic acid tert.-butyl ester

Prepared from [2-amino-5-tert.-butoxy-4-(4-fluoro-phenylethynyl)-phenyl]-carbamic acid tert.-butyl ester (Example L57) (160 mg, 0.4 mmol) and 3-oxo-3-(3-[1,2,3]triazol-1-yl-phenyl)-propionic acid ethyl ester (Example M2) (104 mg, 0.4 mmol) according to the general procedure O. Obtained as a yellow gum (150 mg).

MS (ISP) 612 [(M+H)$^+$].

General Procedure P

Preparation of 4,8-diaryl-1,3-dihydro-benzo[b][1,4]diazepin-2-ones, 4-aryl-8-aroyl-1,3-dihydro-benzo[b][1,4]diazepin-2-ones or 4-aryl-8-arylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-ones

A suspension of the {2-[3-aryl-3-oxo-propionylamino]-4-aryl-phenyl}-carbamic acid tert-butyl ester or {2-[3-aryl-3-oxo-propionylamino]-4-arylethynyl-phenyl}-carbamic acid tert-butyl ester (1.0 mmol) in CH_2Cl_2 (5 mL) [anisole or 1,3-dimethoxybenzene (5–15 mmol) can be added if necessary] was treated with TFA (0.5–5.0 mL) at 0° C. and stirring was continued at 23° C. until tlc indicated complete consumption of the starting material. The solvent was removed in vacuum, the residue treated with little ether, whereupon it crystallized. The solid was stirred with sat. $NaHCO_3$-sol., filtered, washed with H_2O and ether or mixtures of ether/hexane and was dried to give the title compound, which if necessary can be purified by crystallization from THF/CH_2Cl_2/ether/hexane.

General Procedure Q

Preparation of 4-[3-(Amino-4-carbonyl)-phenyl]-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-ones by Pd-catalyzed carbonylative amination of 4-(3-iodophenyl)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one

A solution of the 4-(3-iodophenyl)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one (1.0 mmol), the secondary amine (5.0 mmol), PPh_3 (6 mol %) or dppp (3 mol %), $Pd(OAc)_2$ (3 mol %) and Et_3N (2.0 mmol) in DMF (4 mL) was stirred at 23° C. under CO-atmosphere until tlc indicated complete consumption of the iodide. After dilution with EtOAc, washing with sat. $NaHCO_3$-sol. and brine, the organic phase was dried over Na_2SO_4. Removal of the solvent left a brown oil, which was purified by silica gel column chromatography with hexane/EtOAc to give the title compound.

General Procedure R

Preparation of (5-hydroxy-2-nitro-phenyl)-carbamic acid tert.-butyl esters by Rh-catalyzed deallylation of (5-allyloxy-2-nitro-phenyl)-carbamic acid tert.-butyl esters

A mixture of the (5-allyloxy-2-nitro-phenyl)-carbamic acid tert.-butyl ester, $(PPh_3)_3RhCl$ (5 mol %) and DABCO (20 mol %) in EtOH was refluxed for 2.5 h according to *J. Org. Chem.* 1973, 38, 3224. Added 5% citric acid, stirred at 23° C. for 15 min, extracted with EtOAc, washed with brine, dried over $MgSO_4$. Removal of the solvent left an orange solid, which was purified by silica gel column chromatography with hexane/EtOAc to give the title compound.

Example R1

(5-Hydroxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester

Prepared from (5-allyloxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example B4), $(PPh_3)_3RhCl$ (5 mol %) and DABCO (20 mol %) in EtOH according to the general procedure R. Obtained as a yellow solid.

MS (ISN) 379 [(M–H)$^-$]; mp 140° C.

General Procedure S

Preparation of 5-O-substituted-(4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl esters from (5-hydroxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester

A mixture of the (5-hydroxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example R1), $KHCO_3$ and the appropriate alkylating reagent were stirred in DMF at 23 to 60° C. until tlc indicated complete conversion. Dilution with EtOAc was followed by aqueous workup with 5% citric acid, sat. $NaHCO_3$-sol., brine and drying over $MgSO_4$. Removal of the solvent left a crude material, which was purified by silica gel column chromatography with hexane/EtOAc to give the title compound.

Example S1

(5-tert.-Butoxycarbonylamino-2-iodo-4-nitro-phenoxy)-acetic acid tert.-butyl ester

Prepared from (5-hydroxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example R1) (1.23 g, 3.24 mmol), $KHCO_3$ (0.39 g, 3.89 mmol) and tert.-butyl bromoacetate (0.59 mL, 3.89 mmol) according to the general procedure S. Obtained as a yellow solid (1.5 g, 94%)

MS (ISP) 495 [(M+H)$^+$], 512 [(M+NH$_4$)$^+$] and 517 [(M+Na)$^+$]; mp 103° C.

Example S2

(5-Cyanomethoxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester

Prepared from (5-hydroxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example R1) (614 mg, 1.62 mmol), $KHCO_3$ (208 mg, 2.08 mmol) and bromoacetonitrile

(0.21 mL, 3.16 mmol) according to the general procedure S. Obtained as a yellow solid (574 mg, 85%).

MS (ISN) 418 [(M–H)⁻]; mp 125° C.

Example S3

(RS)-{4-Iodo-2-nitro-5-[2-(tetrahydro-pyran-2-yloxy)-ethoxy]-phenyl}-carbamic acid tert.-butyl ester

Prepared from (5-hydroxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example R1) (760 mg, 2 mmol), $KHCO_3$ (260 mg, 4 mmol) and 2-(2-bromoethoxy) tetrahydro-2H-pyran (0.6 mL, 2.6 mmol) according to the general procedure S. Obtained as an orange oil (804 mg, 79%).

MS (EI) 508 (M^+).

Example S4

(5-tert.-Butoxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester

N,N-Dimethylformamide di-tert.butylacetal (19.2 mL, 80 mmol) was added dropwise within 15 min to a solution of (5-hydroxy-4-iodo-2-nitro-phenyl)-carbamic acid tert.-butyl ester (Example R1) (7.60 g, 20 mmol) in toluene at 80° C. and stirring was continued at 80° C. for 3 h (cf. *Synthesis* 1983, 135). Obtained as a yellow solid (5.97 g, 68%).

MS (ISN) 435 [(M–H)⁻]; mp 94° C.

Example 1

3-(7-Iodo-4-oxo-8-thiomorpholin-4-yl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-benzonitrile

Prepared from 2-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-4-iodo-5-thiomorpholin-4-yl-phenyl}-carbamic acid tert.-butyl ester (Example O1) (629 mg, 1.04 mmol) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as an olive solid (437 mg).

mp 227–228° C. (dec.).

Example 2

3-(7-Iodo-8-morpholin-4-yl-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl-benzonitrile

Prepared from {2-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-4-iodo-5-morpholin-4-yl-phenyl}-carbamic acid tert.-butyl ester (Example O2) (518 mg, 0.877 mmol) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a beige solid (309 mg).

MS (EI) 472 (M^+); mp 224° C. (dec.).

Example 3

3-(8-Chloro-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-benzonitrile

Prepared from (2-amino-5-chloro-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester (Example L3) (171 mg, 0.5 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (183 mg, 0.6 mmol) according to the general procedure O. Obtained as a light yellow solid (284 mg). This material was deprotected and cyclized by treatment with TFA in CH_2Cl2 according to the general procedure P. Obtained as an orange solid (483 mg).

MS (ISP) 343 [(M+H)⁺] and 345 [(M+2+Na)⁺]; mp 248–251° C. (dec.).

Example 4

3-(8-Methyl-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-benzonitrile

Prepared from (2-amino-5-methyl-4-phenylethynyl-phenyl)-carbamic acid tert.-butyl ester (Example L4) (161 mg, 0.5 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (230 mg, 0.75 mmol, 75% pure) according to the general procedure O. Obtained as a light yellow solid (227 mg). This material was deprotected and cyclized by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (83 mg).

MS (ISP) 375 (M^+); mp 237–239° C. (dec.).

Example 5

3-[8-(4-Methyl-piperazin-1-yl)-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4] diazepin-2-yl]-benzonitrile

Prepared from [2-amino-5-(4-methyl-piperazin-1-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example L5) (203 mg, 0.5 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (230 mg, 0.75 mmol, 75% pure) according to the general procedure O. Obtained by chromatography as an orange oil (181 mg). This material was deprotected and cyclized by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as an orange solid (82 mg).

MS (ISP) 460.5 [(M+H)⁺]; mp 222–224° C. (dec.).

Example 6

3-[8-(1,1-Dioxo-thiomorpholin-4-yl)-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4] diazepin-2-yl]-benzonitrile

Prepared from [2-amino-5-(1,1-dioxo-6-thiomorpholin-4-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example L19) (220 mg, 0.5 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-benzonitrile (Example N4) (172 mg, 0.75 mmol) according to the general procedure O. The obtained material was deprotected and cyclized by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (47 mg).

MS (ISP) 495 [(M+H)⁺]; mp>250° C. (dec.).

Example 7

3-(8-Chloro-4-oxo-7-phenyl-4,5-dihydro-3H-benzo [b][1,4]diazepin-2-yl)-benzonitrile

Prepared from {2-chloro-5-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O3) (720 mg, 1.47 mmol) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as an off-white solid (457 mg).

MS (EI) 371 (M^+) and 373 [(M+2)⁺]; mp 242–244° C. (dec.).

Example 8

3-(8-Methyl-4-oxo-7-phenyl-4,5-dihydro-3H-benzo [b][14]diazepin-2-yl)-benzonitrile

Prepared from (5-amino-2-methyl-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example L7) (298 mg, 1.0 mmol) and 3-(2,2-dimethyl-6-oxo-6H-[1,3]dioxin-4-yl)-

benzonitrile (Example N4) (460 mg, 1.5 mmol) according to the general procedure O. The obtained material (351 mg) was deprotected and cyclized by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (206 mg).

MS (EI) 351 (M^+); mp 236–239° C. (dec.).

Example 9

3-[8-(1,4-Dioxa-8-aza-spiro[4.5]dec-8-yl)-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4] diazepin-2-yl]-benzonitrile

Prepared from [2-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-5-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example O14) (265 mg, 0.43 mmol) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a brown solid (121 mg).

MS (ISP) 503 [$(M+H)^+$]; mp 239–243° C. (dec.).

Example 10

3-(8-Morpholin-4-yl-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl-)-benzonitrile

Prepared from {2-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-5-morpholin-4-yl-4-phenylethynyl-phenyl}-carbamic acid tert.-butyl ester O10 (370 mg, 0.66 mmol) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light brown solid (216 mg).

MS (EI) 446 (M^+); mp 239–243° C. (dec.).

Example 11

3-[8-(2-Dimethylamino-ethylsulfanyl)-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4] diazepin-2-yl]-benzonitrile

Prepared from [2-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-5-(2-dimethylamino-ethylsulfanyl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (example O4) (166 mg, 0.28 mmol) by treatment with TFA and anisole in CH2Cl2 according to the general procedure P. Obtained as a light yellow solid (103 mg).

MS (ISP) 465 [$(M+H)^+$]; mp 197° C. (dec.).

Example 12

[4-(3-Cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-ylsulfanyl]-acetic acid methyl ester

Prepared from {5-tert.-butoxycarbonylamino-4-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2-phenylethynyl-phenylsulfanyl}-acetic acid methyl ester (Example O5)(421 mg, 0.72 mmol) by treatment with TFA and anisole in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (309 mg).

MS (ISP) 465 [$(M+H)^+$]; mp 201° C. (dec.).

Example 13

[4-(3-Cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-ylsulfanyl]-acetic acid

A solution of [4-(3-cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-ylsulfanyl]-acetic acid methyl ester (Example 12) (265 mg, 0.57 mmol) and $LiOH.H_2O$ (26 mg, 0.63 mmol) in THF (5 mL), MeOH (1 mL) and H_2O (1 mL) was stirred at 23° C. for 24 h. Obtained as a light yellow solid (257 mg).

MS (ISP) 452 [$(M+H)^+$]; mp 202° C. (dec.).

Example 14

[4-(3-Cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yl]-acetic acid methyl ester

Prepared from {5-tert.-butoxycarbonylamino-4-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2-phenylethynyl-phenyl}-acetic acid methyl ester (Example O6) (846 mg, 1.53 mmol) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (557 mg).

MS (EI) 433 (M^+); mp 236° C. (dec.).

Example 15

[4-(3-Cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7yl]-acetic acid

A solution of $LiOH.H_2O$ (54 mg, 1.28 mmol) in H_2O (2 mL) and MeOH (2 mL) was added to [4-(3-cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4] diazepin-7yl]-acetic acid methyl ester (Example 14) (505 mg, 1.17 mmol) in THF (10 mL) and the reaction mixture was stirred at 23° C. for 48 h. Obtained as a light yellow solid (62 mg).

MS (ISP) 452 [$(M+H)^+$]; mp 248° C. (dec.).

Example 16

4-(3-Cyano-phenyl)-8-iodo-2-oxo-2,3-dihydro-1H-benzo[b][1,4]diazepine-7-carboxyl acid methyl ester

Prepared from 5-tert.-butoxycarbonylamino-4-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2-iodo-benzoic acid methyl ester (Example O17) (430 mg, 0.763 mmol) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a salmon colored solid (199 mg).

MS (EI) 445 (M^+); mp 247–248° C. (dec.).

Example 17

2-[4-(3-Cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yl]-acetamide

[4-(3-Cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yl]-acetic acid (Example 15) (48 mg, 0.114 mmol) was treated with Boc_2O (37 mg), NH_4HCO_3 (13 mg) and pyridine (6 μL) in DMF (0.6 mL) at 23° C. for 24 h [cf *Tetrahedron Letters* 1995, 36, 7115]. Obtained as a light yellow solid (14 mg).

MS (ISN) 417 [$(M-H)^-$]; mp 250° C. (dec.).

Example 18

3-[8-(2-Methoxy-ethoxy)-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile

Prepared from [2-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-5-(2-methoxy-ethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example O7) (135 mg, 0.251 mmol) by treatment with TFA in CH_2Cl_2 accord

ing to the general procedure P. Obtained as a light green solid (82 mg).

MS (EI) 435 (M^+); mp 174–176° C.

Example 19

4-(3-Cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepine-7-carboxylic acid methyl ester

Prepared from 5-tert.-butoxycarbonylamino-4-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2-phenylethynyl-benzoic acid methyl ester (Example O9) (511 mg, 0.95 mmol) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as an off-white solid (321 mg).

MS (EI) 419 (M^+); mp>250° C.

Example 20

3-(8-Methoxy-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-benzonitrile

Prepared from {2-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-5-methoxy-4-phenylethynyl-phenyl}-carbamic acid tert.-butyl ester (Example O8) (359 mg, 0.7 mmol) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow-brown solid (87 mg).

MS (EI) 391 (M^+); mp>250° C.

Example 21

3-[8-(2-{2-[2-(2-Methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile

Prepared from [2-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-5-(2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example O11) (300 mg, 0.437 mmol) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (211 mg).

MS (EI) 435 (M^+); mp 140–141° C. (dec.).

Example 22

[4-(3-Cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yloxy]-acetic acid

Prepared from {5-tert.-butoxycarbonylamino-4-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2-phenylethynyl-phenoxy}-acetic acid tert.-butyl ester (Example O12) (698 mg, 1.14 mmol) by treatment with TFA in CH_2Cl_2 and anisole according to the general procedure P. Obtained as a yellow solid (265 mg).

MS (ISN) 434 [$(M-H)^-$]; mp 257° C. (dec.).

Example 23

3-(8-Cyanomethyl-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-benzonitrile

Prepared from {5-cyanomethyl-2-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-4-phenylethynyl-phenyl}-carbamic acid tert.-butyl ester (Example O13) (266 mg, 0.51 mmol) by treatment with TFA in CH_2Cl_2 and anisole according to the general procedure P. Obtained as a yellow solid (145 mg).

MS (EI) 400 (M^+); mp 262° C. (dec.).

Example 24

3-[8-(2,3-Dihydroxy-propoxy)-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile

Prepared from [2-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-5-(2,2-dimethyl-[1,3]dioxolan-4-ylmethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example O16) (265 mg, 0.435 mmol) by treatment with TFA in CH_2Cl_2 and anisole according to the general procedure P. Obtained as a yellow solid (62 mg).

MS (ISP) 452 [$(M+H)^+$] and 474 [$(M+Na)^+$]; mp 230–234° C. (dec.).

Example 25

4-(3-Iodo-phenyl)-7-(2-methoxy-ethoxy)-8-phenylethynyl-1,3-dihydro-benzo[b]1,4]diazepin-2-one

Prepared from [2-[3-(3-iodo-phenyl)-3-oxo-propionylamino]-5-(2-methoxy-ethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example O15) (1.24 g, 1.89 mmol) by treatment with TFA in CH_2Cl_2 and anisole according to the general procedure P. Obtained as a yellow solid (517 mg).

MS (EI) 536 (M^+); mp 192° C. (dec.).

Example 26

2-[4-(3-Cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yloxy]-acetamide

EDC (42 mg, 0.22 mmol) was added to [4-(3-cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yloxy]-acetic acid (Example 22) (50 mg, 0.11 mmol), NH_4Cl (18 mg, 0.33 mmol) and NMM (56 mg, 0.55 mmol) in DMF (1.1 mL) at 0° C. and the reaction mixture was stirred at 23° C. for 2 h. Obtained as a yellow solid (5 mg).

MS (ISN) 417 [$(M-H)^-$]; mp 250° C. (dec.).

Example 27

4-(3-Imidazol-1-yl-phenyl)-7-(2-methoxy-ethoxy)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from [[[2-[3-(3-Imidazol-1-yl-phenyl)-3-oxo-propionylamino]-5-(2-methoxy-ethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example O18) (200 mg, 0.336 mmol) by treatment with TFA in CH_2Cl_2 and anisole according to the general procedure P. Obtained as a yellow solid (28 mg).

MS (EI) 476 (M^+); mp 187–189° C.

Example 28

[RS]-3-[4-Oxo-8-(2-oxo-[1,3]dioxolan-4-ylmethoxy)-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile

Prepared from [RS]-[2-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-5-(2-oxo-[1,3]dioxolan-4-ylmethoxy)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example O19) (400 mg, 0.67 mmol) by treatment with TFA

in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (287 mg).

MS (EI) 477 (M^+); mp 222° C. (dec.).

Example 29

3-(8-Ethoxymethyl-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo [b][1,4]diazepin-2-yl)-benzonitrile

Prepared from {2-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-5-ethoxymethyl-4-phenylethynyl-phenyl}-carbamic acid tert.-butyl ester (Example O20) (140 mg, 0.26 mmol) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (93 mg).

MS (EI) 419 (M^+); mp 229° C.

Example 30

2,2-Dimethyl-propionic acid 4-(3-cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4] diazepin-7-ylmethyl ester

Prepared from 2,2-dimethyl-propionic acid 5-tert.-butoxycarbonylamino-4-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2-phenylethynyl-benzyl ester (Example O21) (156 mg, 0.26 mmol) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (75 mg).

MS (EI) 475 (M^+); mp 218° C.

Example 31

3-(8-Hydroxymethyl-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-benzonitrile

Prepared from 2,2-dimethyl-propionic acid 4-(3-cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-ylmethyl ester (Example 30) (30 mg, 0.063 mmol) and $LiOH.H_2O$ (8 mg, 0.289 mmol) in THF (2 mL), MeOH (0.4 mL) and H_2O (0.4 mL) at 23° C. for 3 days. Obtained as a yellow solid (17 mg).

MS (EI) 391 (M^+); mp >255° C.

Example 32

7-Hydroxymethyl-4-(3-imidazol-1-yl-phenyl)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from (RS)-[2-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-4-phenylethynyl-5-(tetrahydro-pyran-2-yloxymethyl)-phenyl]-carbamic acid tert.-butyl ester (Example O22) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (77 mg).

MS (EI) 432 (M^+); mp 227° C.

Example 33

4-(3-Imidazol-1-yl-phenyl)-7-(4-methoxy-piperidin-1-yl)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4] diazepin-2-one

Prepared from [2-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-5-(4-methoxy-piperidin-1-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example O23) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (159 mg).

MS (ISP) 516 [$(M+H)^+$]; mp 222° C.

Example 34

3-[8-(4-Methoxy-piperidin-1-yl)-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4] diazepin-2-yl]-benzonitrile

Prepared from [2-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-5-(4-methoxy-piperidin-1-yl)-4-phenylethynyl-phenyl]-carbamic acid tert.-butyl ester (Example O24) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (128 mg).

MS (ISP) 475 [$(M+H)^+$]; mp 250–251° C.

Example 35

[4-(3-Imidazol-1-yl-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yloxy]-acetonitrile

Prepared from {5-cyanomethoxy-2-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-4-phenylethynyl-phenyl}-carbamic acid tert.-butyl ester (Example O25) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (43 mg).

MS (EI) 457 (M^+); mp 214° C.

Example 36

3-(8-Cyanomethoxy-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-benzonitrile

Prepared from {5-cyanomethoxy-2-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-4-phenylethynyl-phenyl}-carbamic acid tert.-butyl ester (Example O26) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (71 mg).

MS (EI) 416 (M^+); mp 212° C.

Example 37

8-(4-Fluoro-phenylethynyl)-7-hydroxymethyl-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4] diazepin-2-one

Prepared from (RS)-[4-(4-fluoro-phenylethynyl)-2-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-5-(tetrahydro-pyran-2-yloxymethyl)-phenyl]-carbamic acid tert.-butyl ester (Example O27) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (462 mg).

MS (EI) 450 (M^+); mp 234° C. (dec.).

Example 38

8-(4-Fluoro-phenylethynyl)-7-[(2-hydroxy-ethyl)-methyl-amino]-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from (RS)-(4-(4-fluoro-phenylethynyl)-2-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-5-{methyl-[2-(tetrahydro-pyran-2-yloxy)-ethyl]-amino}-phenyl)-carbamic acid tert.-butyl ester (Example O28) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (73 mg).

MS (EI) 493 (M^+); mp 217° C. (dec.).

Example 39

4-(3-Imidazol-1-yl-phenyl)-7-(4-oxo-piperidin-1-yl)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from {5-(4,4-diethoxy-piperidin-1-yl)-2-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-4-

phenylethynyl-phenyl}-carbamic acid tert.-butyl ester (Example O29) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (180 mg).

MS (EI) 499 (M^+); mp 231° C. (dec.).

Example 40

N-tert.-Butyl-2-[4-(3-cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4] diazepin-7-yloxy]-acetamide

Prepared from [4-(3-cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yloxy]-acetic acid (Example 22) (87 mg, 0.2 mmol) by treatment with oxalyl chloride (26 uL, 0.3 mmol) in DMF (0.6 mL) at 0° C. for 1 h, then with tert.-butylamine (106 uL, 1.0 mmol) at 0° C. for further 30 min. Obtained as a yellow solid (21 mg).

MS (EI) 490 (M^+); mp>250° C.

Example 41

2-[4-(3-Cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yloxy]-N-methoxy-acetamide

Prepared from [4-(3-cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yloxy]-acetic acid (Example 22) (44 mg, 0.1 mmol) by treatment with EDC (38 mg, 0.2 mmol), MeONH2.HCl (9 mg, 0.11 mmol), NMM (0.021 mL, 0.3 mmol) and HOBt (15 mg, 0.11 mmol) in DMF (1 mL) at 0 to 23° C. for 20 h. Obtained as a yellow solid (36 mg).

MS (ISP) 465 [$(M+H)^+$]; mp>250° C.

Example 42

7-(2-Hydroxy-ethoxy)-4-(3-imidazol-1-yl-phenyl)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from (RS)-{2-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-4-phenylethynyl-5-[2-(tetrahydro-pyran-2-yloxy)-ethoxy]-phenyl}-carbamic acid tert.-butyl ester (Example O29) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (48 mg).

MS (EI) 462 (M^+); mp 224–227° C.

Example 43

8-(4-Fluoro-phenyl)-7-(4-hydroxy-piperidin-1-yl)-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4] diazepin-2-one

Prepared from (RS)-{4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-[4-(tetrahydro-pyran-2-yloxy)-piperidin-1-yl]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O31) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (109 mg).

MS (ISP) 496 [$(M+H)^+$]; mp 238–240° C.

Example 44

8-(4-Fluoro-phenylethynyl)-7-(2-hydroxy-ethoxy)-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4] diazepin-2-one

Prepared from {5-(2-tert.-butoxy-ethoxy)-4-(4-fluoro-phenylethynyl)-2-[3-(3-imidazol-1-yl -phenyl)-3-oxo-propionylamino]-phenyl}-carbamic acid tert.-butyl ester (Example O32) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (83 mg).

MS (EI) 480 (M^+); mp 220–222° C.

Example 45

8-(4-Fluoro-phenyl)-7-[4-(2-hydroxy-ethoxy)-piperidin-1-yl]-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from (RS)-(4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-{4-[2-(tetrahydro-pyran-2-yloxy)-ethoxy]-piperidin-1-yl}-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example O33) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (97 mg).

MS (ISP) 540 [$(M+H)^+$]; mp 225–227° C.

Example 46

8-(4-Fluoro-phenyl)-7-hydroxymethyl-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4] diazepin-2-one

Prepared from (RS)-[4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-(tetrahydro-pyran-2-yloxymethyl)-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example O34) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (]62 mg).

MS (EI) 426 [$(M)^+$]; mp 180–195° C.

Example 47

[8-(4-Fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-2-oxo-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yloxy]-acetonitrile

Prepared from {2-cyanomethoxy-4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O35) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (11 mg).

MS (EI) 451 (M^+); mp 164° C.

Example 48

7-Dimethylaminomethyl-8-(4-fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4] diazepin-2-one

Prepared from {2-dimethylaminomethyl-4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O36) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a brown solid (180 mg).

MS (ISP) 454 $(M+H)^+$; mp 115–140° C. (dec.).

Example 49

7-(2,2-Dimethyl-tetrahydro-[1,3]dioxolo [4,5-c] pyrrol-5-yl)-8-(4-fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from {2-(2,2-dimethyl-tetrahydro-[1,3]dioxolo [4,5-c]pyrrol-5-yl)-4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-

butyl ester (Example O37) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (358 mg).

MS (EI) 537 (M^+); mp 240° C. (dec.).

Example 50

7-(cis-3,4-Dihydroxy-pyrrolidin-1-yl)-8-(4-fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from 7-(2,2-dimethyl-tetrahydro-[1,3]dioxolo [4,5-c]pyrrol-5-yl)-8-(4-fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one (Example 49) (304 mg, 0.57 mmol) by treatment 13% HCl (15 mL) in THF (50 mL) at 23° C. for 16 h. Obtained as a yellow solid (209 mg).

MS (ISP) 498 [$(M+H)^+$]; mp 244° C.

Example 51

8-(4-Fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-7-methoxy-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from {4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-methoxy-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O38) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (182 mg).

MS (EI) 426 (M^+); mp 221° C. (dec.).

Example 52

8-(4-Fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-7-(4-oxo-piperidin-1-yl)-1,3-dihydro-benzo[b][1,4] diazepin-2-one

Prepared from {2-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O39) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as an orange-brown solid (150 mg).

MS (ISP) 494 [$(M+H)^+$]; mp 204° C.

Example 53

8-(4-Fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-7-methyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from {4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-methyl-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O40) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (216 mg).

MS (EI) 410 (M^+); mp 196° C.

Example 54

[8-(4-Fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-2-oxo-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yloxy-acetic acid

Prepared from {4-tert.-butoxycarbonylamino-4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-2-yloxy}-acetic acid tert.-butyl ester (Example O41) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a beige solid (570 mg).

MS (ISP) 471 [$(M+H)^+$]; mp 209° C. (dec.).

Example 55

2-[8-(4-Fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-2-oxo-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yloxy-N-hydroxy-acetamide

Prepared from [8-(4-fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-2-oxo-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yloxy]-acetic acid (Example 54) (94 mg, 0.2 mmol) by reaction with O-tritylhydroxylamine (61 mg, 0.22 mmol), HOBT (30 mg, 0.22 mmol), N-methylmorpholine (66 μL, 0.6 mmol) and EDC (77 mg, 0.4 mmol) in DMF (2 mL) from 0 to 23° C. for 18 h. After extraction and chromatography the resulting orange solid was stirred with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (71 mg).

MS (ISP) 486 [$(M+H)^+$]; mp 147–157° C. (dec.).

Example 56

7-Chloro-8-(4-fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from {2-chloro-4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O42) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (35 mg).

MS (EI) 430 ((M^+); mp 209–211° C.

Example 57

8-(4-Fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-7-(2-methoxy-ethoxy)-1,3-dihydro-benzo[b][1,4] diazepin-2-one

Prepared from [4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-(2-methoxy-ethoxy)-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example O43) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (96 mg).

MS (EI) 470 ((M^+); mp 196–197° C.

Example 58

8-(4-Fluoro-phenyl)-7-(2-hydroxy-ethoxy)-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4] diazepin-2-one

Prepared from {2-(2-tert.-butoxy-ethoxy)-4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O44) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light green solid (95 mg).

MS (EI) 456 (M^+); mp 225° C.

Example 59

8-(4-Fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-7-(2-oxo-oxazolidin-3-yl)-1,3-dihydro-benzo[b][1,4] diazepin-2-one

Prepared from [4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-(2-oxo-oxazolidin-3-yl)-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example O45) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (35 mg).

MS (EI) 481 (M^+); mp 230° C.

Example 60

8-(4-Fluoro-2-methyl-phenyl)-4-(3-imidazol-1-yl-phenyl)-7-methoxy-1,3-dihydro-benzo[b][1,4] diazepin-2-one

Prepared from {4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-methoxy-2'-methyl-biphenyl-4-

yl}-carbamic acid tert.-butyl ester (Example O46) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a beige solid (101 mg).

MS (EI) 440 (M^+); mp 225° C.

Example 61

8-(4-Fluoro-phenyl)-7-hydroxy-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from {2-tert.-butoxy-4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O47) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (109 mg).

MS (EI) 412 (M^+); mp 250° C.

Example 62

8-(2-Fluoro-phenyl)-7-hydroxy-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from {2-tert.-butoxy-2'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O48) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (132 mg).

MS (EI) 412 ((M^+); mp 220° C.

Example 63

8-(4-Fluoro-phenyl)-7-((R)-3-hydroxy-pyrrolidin-1-yl)-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from (RS)-{4'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-[(R)-3-(tetrahydro-pyran-2-yloxy)-pyrrolidin-1-yl]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O49) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (74 mg).

MS (EI) 481 (M^+); mp 155–158° C.

Example 64

8-(2-Fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-7-methoxy-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from {2'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-2-methoxy-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O50) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (68 mg).

MS (EI) 426 ((M^+); mp 216° C. (dec.).

Example 65

3-[7-(4-Fluoro-phenyl)-8-hydroxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile

Prepared from {2-tert.-butoxy-5-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-4'-fluoro-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O51) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (66 mg).

MS (EI) 371 ((M^+); mp>250° C.

Example 66

3-[7-(2-Fluoro-phenyl)-8-hydroxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile

Prepared from {2-tert.-butoxy-5-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2'-fluoro-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O52) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (80 mg).

MS (EI) 371 ((M^+); mp>250° C.

Example 67

3-[7-(2-Fluoro-phenyl)-8-methoxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile

Prepared from {5-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2'-fluoro-2-methoxy-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O53) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (51 mg).

MS (EI) 385 (M^+); mp 245–247° C.

Example 68

8-(2-Fluoro-phenyl)-7-methoxy-4-[3-(2-methyl-imidazol-1-yl)-phenyl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from (2'-fluoro-2-methoxy-5-{3-[3-(2-methyl-imidazol-1-yl)-phenyl]-3-oxo-propionylamino}-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example O54) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (207 mg).

MS (EI) 440 (M^+); mp 220–222° C.

Example 69

5-[7-(2-Fluoro-phenyl)-8-methoxy-4-oxo-4,5-dihydro-3H-benzo b][1,4]diazepin-2-yl]-thiophene-2-carbonitrile

Prepared from {5-[3-(5-cyano-thiophen-2-yl)-3-oxo-propionylamino]-2'-fluoro-2-methoxy-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O55) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (103 mg).

MS (EI) 391 (M^+); mp>250° C.

Example 70

8-(2-Fluoro-phenyl)-7-methoxy-4-(3-[1,2,4]triazol-4-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from {2'-fluoro-2-methoxy-5-[3-oxo-3-(3-[1,2,4]triazol-4-yl-phenyl)-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O56) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (22 mg).

MS (EI) 427 (M^+); mp 188° C. (dec.).

Example 71

4-[7-(2-Fluoro-phenyl)-8-methoxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-pyridine-2-carbonitrile

Prepared from {5-[3-(2-cyano-pyridin-4-yl)-3-oxo-propionylamino]-2'-fluoro-2-methoxy-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O57) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (68 mg).

MS (EI) 386 (M^+); mp 240–242° C.

Example 72

8-(4-Fluoro-phenyl)-7-hydroxy-4-[3-(2-methyl-imidazol-l -yl)-phenyl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from (2-tert.-butoxy-4'-fluoro-5-{3-[3-(2-methyl-imidazol-1-yl)-phenyl]-3-oxo-propionylamino}-

biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example O58) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (49 mg).

MS (ISP) 427 [(M+H)$^+$]; mp 260° C.

Example 73

4-Fluoro-3-[7-(2-fluoro-phenyl)-8-methoxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile

Prepared from {5-[3-(5-cyano-2-fluoro-phenyl)-3-oxo-propionylamino]-2'-fluoro-2-methoxy-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O59) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (52 mg).

MS (ISP) 404 [(M+H)$^+$]; mp>250° C.

Example 74

4-[7-(4-Fluoro-phenyl)-8-hydroxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-pyridine-2-carbonitrile

Prepared from {2-tert.-butoxy-5-[3-(2-cyano-pyridin-4-yl)-3-oxo-propionylamino]-4'-fluoro-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O60) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (24 mg).

MS (EI) 372 (M$^+$); mp 164° C.

Example 75

8-(2-Fluoro-phenyl)-7-hydroxy-4-(3-[1,2,3]triazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from {2-tert.-butoxy-2'-fluoro-5-[3-oxo-3-(3-[1,2,3]triazol-1-yl-phenyl)-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O61) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (61 mg).

MS (ISP) 414 [(M+H)$^+$]; mp>250° C.

Example 76

3-[8-(1,4-Dioxa-8-aza-spiro[4.5]dec-8-yl)-7-(2-fluoro-phenyl)-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile

Prepared from [5-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-2'-fluoro-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example O62) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (132 mg).

MS (ISP) 497 [(M+H)$^+$]; mp 253° C.

Example 77

7-(1,4-Dioxa-8-aza-spiro[4.5]dec-8-yl)-8-(2-fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from {2-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-2'-fluoro-5-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O63) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (133 mg).

MS (ISP) 538 [(M+H)$^+$]; mp 225° C.

Example 78

8-(2-Fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-7-(4-oxo-piperidin-1-yl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from 7-(1,4-dioxa-8-aza-spiro[4.5]dec-8-yl)-8-(2-fluoro-phenyl)-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one (Example 77) (54 mg, 0.1 mmol) by stirring in 1N HCl (1 mL) and acetone (1 mL) at 23° C. for 44 h. Obtained as a yellow solid (39 mg).

MS (EI) 493 (M$^+$); mp 230° C.

Example 79

8-(2-Fluoro-phenyl)-7-hydroxy-4-[3-(2-methyl-imidazol-1-yl)-phenyl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from (2-tert.-butoxy-2'-fluoro-5-{3-[3-(2-methyl-imidazol-1-yl)-phenyl]-3-oxo-propionylamino}-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example O64) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (111 mg).

MS (ISN) 425 [(M−H)$^-$]; mp>250° C.

Example 80

4-[7-(2-Fluoro-phenyl)-8-hydroxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-pyridine-2-carbonitrile

Prepared from {2-tert.-butoxy-5-[3-(2-cyano-pyridin-4-yl)-3-oxo-propionylamino]-2'-fluoro-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O65) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (47 mg).

MS (ISN) 371 [(M−H)$^-$]; mp>250° C.

Example 81

8-(2-Fluoro-phenyl)-7-hydroxy-4-[3-(2-methylsulfanyl-imidazol-1-yl)-phenyl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from (2-tert.-butoxy-2'-fluoro-5-{3-[3-(2-methylsulfanyl-imidazol-1-yl)-phenyl]-3-oxo-propionylamino}-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example O66) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (148 mg).

MS (ISN) 457 [(M−H)$^-$]; mp>250° C.

Example 82

3-[7-(2,5-Difluoro-phenyl)-8-methoxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile

Prepared from {5-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2',5'-difluoro-2-methoxy-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O67) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (49 mg).

MS (EI) 403 (M$^+$); mp 251° C.

Example 83

8-(2,5-Difluoro-phenyl)-7-methoxy-4-(3-[1,2,3]triazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from {2',5'-difluoro-2-methoxy-5-[3-oxo-3-(3-[1,2,3]triazol-1-yl-phenyl)-propionylamino]-biphenyl-4-

yl}-carbamic acid tert.-butyl ester (Example O68) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (78 mg).

MS (EI) 445 (M^+); mp 241° C.

Example 84

2-[7-(2-Fluoro-phenyl)-8-hydroxy-4-oxo-4,5-dihydro-1H-benzo[b][1,4]diazepin-2yl]-thiophene-3-carbonitrile

Prepared from {2-tert.-butoxy-5-[3-(3-cyano-thiophen-2-yl)-3-oxo-propionylamino]-2'-fluoro-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O69) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as an orange solid (82 mg).

MS (ISN) 376 [$(M-H)^-$]; mp 242° C.

Example 85

5-[7-(2-Fluoro-phenyl)-8-hydroxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-thiophene-2-carbonitrile

Prepared from {2-tert.-butoxy-5-[3-(5-cyano-thiophen-2-yl)-3-oxo-propionylamino]-2'-fluoro-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O70) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (126 mg).

MS (EI) 377 (M^+); mp.

Example 86

8-(4-Fluoro-phenyl)-7-hydroxy-4-(3-[1,2,3]triazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from {2-tert.-butoxy-4'-fluoro-5-[3-oxo-3-(3-[1,2,3]triazol-1-yl-phenyl)-propionylamino]-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O71) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (78 mg).

MS (ISP) 414 [$(M+H)^+$]; mp>250° C.

Example 87

3-[7-(2-Fluoro-phenyl)-8-(2-methoxy-ethoxy)-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile

Prepared from [5-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2'-fluoro-2-(2-methoxy-ethoxy)-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example O72) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (141 mg).

MS (EI) 429 (M^+); mp 211–213° C.

Example 88

3-[7-(2-Fluoro-phenyl)-8-hydroxymethyl-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile

Prepared from (RS)-[5-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2'-fluoro-2-(tetrahydro-pyran-2-yloxymethyl)-biphenyl-4-yl]-carbamic acid tert.-butyl ester (Example O73) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a light yellow solid (69 mg).

MS (EI) 385 (M^+); mp 90–91° C.

Example 89

8-(2-Fluoro-phenyl)-7-hydroxy-4-[3-(3-methyl-isoxazol-5-yl)-phenyl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from (2'-fluoro-2-(4-methoxy-benzyloxy)-5-{3-[3-(3-methyl-isoxazol-5-yl)-phenyl]-3-oxo-propionylamino}-biphenyl-4-yl)-carbamic acid tert.-butyl ester (Example O74) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (278 mg).

MS (ISP) 428 ($M+H)^+$; mp 237° C.

Example 90

3-[7-(2,5-Difluoro-phenyl)-8-hydroxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile

Prepared from {2-tert.-butoxy-5-[3-(3-cyano-phenyl)-3-oxo-propionylamino]-2',5'-diflouro-biphenyl-4-yl}-carbamic acid tert.-butyl ester (Example O75) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (56 mg).

MS (ISP) 390 ($M+H)^+$; mp>250° C.

Example 91

8-(4-Fluoro-phenylethynyl)-7-hydroxy-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from {5-tert.-butoxy-4-(4-fluoro-phenylethynyl)-2-[3-(3-imidazol-1-yl-phenyl)-3-oxo-propionylamino]-phenyl}-carbamic acid tert.-butyl ester (Example O76) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (55 mg).

MS (EI) 436 (M^+); mp 247° C.

Example 92

8-(4-Fluoro-phenylethynyl)-7-hydroxy-4-(3-[1,2,3]triazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one

Prepared from {5-tert.-butoxy-4-(4-fluoro-phenylethynyl)-2-[3-oxo-3-(3-[1,2,3]triazol-1-yl-phenyl)-propionylamino]-phenyl}-carbamic acid tert.-butyl ester (Example O77) by treatment with TFA in CH_2Cl_2 according to the general procedure P. Obtained as a yellow solid (56 mg).

MS (EI) 437 (M^+); mp 243° C.

The following examples exemplify, that the 4-aryl-8-iodo-1,3-dihydro-benzo[b][1,4]diazepin-2-ones could also serve as starting material for the Sonogashira-coupling as illustrated in synthetic scheme G.

Example 93

3-(4-Oxo-7-phenylethynyl-8-thiomorpholin-4-yl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-benzonitrile

Prepared from 3-(7-iodo-4-oxo-8-thiomorpholin-4-yl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-benzonitrile (Example 1) (437 mg, 0.895 mmol) and phenylacetylene (0.15 mL, 1.34 mmol) according to the general procedure K. Obtained as a curry solid (334 mg).

MS (EI) 391 (M^+); mp 234–235° C. (dec.).

Example 94

(RS)-3-[4-Oxo-8-(1-oxo-thiomorpholin-4-yl)-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile

A mixture of 3-(4-oxo-7-phenylethynyl-8-thiomorpholin-4-yl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-

benzonitrile (Example 27) (50 mg, 0.180 mmol) and Davis-reagent (116 mg, 0.432 mmol) in DCM (4.5 mL) was stirred at 23° C. for 1 h. The product was filtered off and washed with DCM. Obtained as a light yellow solid (16 mg).

MS (ISP) 479 [(M+H)$^+$] and 501 [(M+Na)$^+$]; mp>250° C. (dec.).

Palladium-catalyzed carbonylation of the 4-(3-iodophenyl)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4] diazepin-2-one in the presence of secondary amines leads directly to the corresponding amides as shown in synthetic scheme I.

Example 95

3-[8-(2-Methoxy-ethoxy)-4-oxo-7-phenylethynyl-4, 5-dihydro-3H-benzo[b][1,4]diazepin 2-yl]-benzamide

Prepared from 4-(3-iodo-phenyl)-7-(2-methoxy-ethoxy)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one (Example 25) (268 mg, 0.5 mmol) and hexamethyldisilazane (0.52 mL, 2.5 mmol) according to the general procedure Q. Obtained as a yellow solid (102 mg).

MS (EI) 453 ((M$^+$); mp 227–230° C. (dec.).

Example I

Tablets of the following composition are produced in a conventional manner:

	mg/Tablet
Active ingredient	100
Powdered lactose	95
White corn starch	35
Polyvinylpyrrolidone	8
Na carboxymethylstarch	10
Magnesium stearate	2
Tablet weight	250

Example II

Tablets of the following composition are produced in a conventional manner:

	mg/Tablet
Active ingredient	200
Powdered lactose	100
White corn starch	64
Polyvinylpyrrolidone	12
Na carboxymethylstarch	20
Magnesium stearate	4
Tablet weight	400

Example III

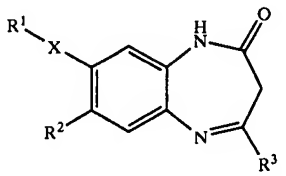
Capsules of the following composition are produced:

	mg/Capsule
Active ingredient	50
Crystalline lactose	60
Microcrystalline cellulose	34
Talc	5
Magnesium stearate	1
Capsule fill weight	150

The active ingredient having a suitable particle size, the crystalline lactose and the microcrystalline cellulose are homogeneously mixed with one another, sieved and thereafter talc and magnesium stearate are admixed. The final mixture is filled into hard gelatine capsules of suitable size.

What is claimed is:

1. A compound of the formula



I

wherein

X is a single bond or an ethynediyl group, wherein,

when X is a single bond, R^1 is halogen or phenyl which is optionally substituted with halogen, lower alkyl, halo-lower alkyl, lower alkoxy, halo-lower alkoxy, or cyano; or

when X is an ethynediyl group, R^1 is unsubstituted phenyl, or phenyl substituted with halogen, lower alkyl, halo-lower alkyl, lower cycloalkyl, lower alkoxy or halo-lower alkoxy;

R^2 is halogen, hydroxy, lower alkyl, lower halo-alkyl, lower alkoxy, hydroxymethyl, hydroxyethoxy, lower alkoxy-(ethoxy)$_n$(n=1 to 4), lower alkoxymethyl, cyanomethoxy, morpholine-4-yl, thiomorpholine-4-yl, 1-oxothiomorpholine-4-yl, 1,1-dioxothiomorpholine-4-yl, 4-oxo-piperidine-1-yl, 4-alkoxy-piperidine-1-yl, 4-hydroxy-piperidine-1-yl, 4-hydroxyethoxy-piperidine-1-yl, 4-lower alkyl-piperazine-1-yl, alkoxycarbonyl, 2-dialkylamino-ethylsulfanyl-, N,N-bis lower alkylamino lower alkyl; carbamoylmethyl; alkylsulfonyl; lower alkoxycarbonyl-lower alkyl, alkylcarboxy-lower alkyl, carboxy-lower alkyl, alkoxycarbonylmethylsulfanyl, carboxymethylsulfanyl, 1,4-dioxa-8-aza-spiro[4,5]dec-8-yl, carboxy-lower alkoxy, cyano-lower alkyl, 2,3-dihydroxy-lower alkoxy, carbamoylmethoxy, 2-oxo-[1,3]-dioxolan-4-yl-lower alkoxy, (2-hydroxy-lower alkyl)-lower alkyl amino, hydroxycarbamoyl-lower alkoxy, 2,2-dimethyl-tetrahydro-[1,3]dioxolo[4,5c]-pyrrol-5-yl, lower alkoxy-carbamoyl-lower alkoxy, 3 R-hydroxy-pyrrolidin-1-yl, 3,4-dihydroxy-pyrrolidin-1-yl, 2-oxo-oxazolidin-3-yl, lower alkyl-carbamoylmethoxy, or aminocarbamoyl-lower alkoxy; and

R^3 is an unsubstituted 5 or 6 membered aryl or heteroaryl or a substituted 5 or 6 membered aryl or heteroaryl with a substituent selected from the group consisting of

halogen, cyano, nitro, azido, hydroxy, carboxy, morpholine-4-carbonyl, carbamoyl, thiocarbamoyl, N-hydroxycarbamoyl, trimethylsilyl-ethynyl, lower alkyl, lower alkoxy, halo-lower alkyl, 4-lower alkyl-piperazine-1-carbonyl,

unsubstituted lower alkylcarbamoyl or lower alkylcarbamoyl substituted by amino, lower alkylamino, acylamino, oxo, hydroxy, lower alkoxy, lower alkylthio,

unsubstituted carboxy or an esterified or amidated carboxy,

unsubstituted five-membered aromatic heterocycle, or a five-membered aromatic heterocycle substituted by amino, lower alkylamino, acylamino, oxo, hydroxy, lower alkoxy, lower alkylthio,

unsubstituted carboxy or an esterified or amidated carboxy,

unsubstituted lower alkyl or a lower alkyl substituted by halogen, amino, lower alkylamino, acylamino, hydroxy, lower alkoxy, lower alkylthio, acyloxy, lower alkenoyl, lower alkylsulfinyl, lower alkylsulfonyl, cycloalkylsulfinyl, cycloalkylsulfonyl, hydroxyimino, alkoxyimino,

unsubstituted carboxy or an esterified or amidated carboxy,

lower alkenyl, oxo, cyano, carbamoyloxy,

unsubstituted sulfamoyl or sulfamoyl substituted by lower alkyl, and

unsubstituted amidino or amidino substituted by lower alkyl, -C(NRR')=NR" (where R, R' and R" are hydrogen or lower alkyl),

or a pharmaceutically acceptable addition salt thereof.

2. The compound according to claim **1**, wherein R^3 is phenyl substituted in the meta position by a substituent selected from the group consisting of cyano, halogen, unsubstituted imidazolyl or imidazolyl substituted by lower alkyl or methylsulfanyl, 1,2,3-triazolyl, 1,2,4-triazolyl, unsubstituted isoxazolyl and isoxazolyl substituted by lower alkyl.

3. The compound according to claim **2**, selected from the group consisting of

3-(8-Chloro-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo [b](1,4]diazepin-2-yl)-benzonitrile;

3-[8-(4-Methyl-piperazin-1-yl)-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile;

3-(8-Chloro-4-oxo-7-phenyl-4,5-dihydro-3H -benzo[b][1,4] diazepin-2-yl)-benzonitrile;

[4-(3-Cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-ylsulfanyl]-acetic acid methyl ester;

2-[4-(3-Cyano-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yl]-acetamide;

3-(8-Methoxy-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-benzonitrile;

3-(8-Cyanomethyl-4-oxo-7-phenylethynyl-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl)-benzonitrile;

4-(3-Iodo-phenyl)-7-(2-methoxy-ethoxy)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

4-(3-Imidazol-1-yl-phenyl)-7-(2-methoxy-ethoxy)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

[RS]-3-[4-Oxo-8-(2-oxo-[1,3]dioxolan-4-ylmethoxy)-7-phenylethynyl-4,5-dihydro3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile;

7-Hydroxymethyl-4-(3-imidazol-1-yl-phenyl)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

[4-(3-Imidazol-1-yl-phenyl)-2-oxo-8-phenylethynyl-2,3-dihydro-1H-benzo[b][1,4]diazepin-7-yloxy]-acetonitrile;

8-(4-Fluoro-phenylethynyl)-7-hydroxymethyl-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4] diazepin-2-one;

7-(2-Hydroxy-ethoxy)-4-(3-imidazol-1-yl-phenyl)-8-phenylethynyl-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

8-(4-Fluoro-phenyl)-7-[4-(2-hydroxy-ethoxy)-piperidin-1-yl]-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

8-(4-Fluoro-phenyl)-7-hydroxy-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

8-(2-Fluoro-phenyl)-7-methoxy-4-[3-(2-methyl-imidazol-1-yl)-phenyl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

8-(2-Fluoro-phenyl)-7-hydroxy-4-(3-[1,2,3]triazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

8-(2-Fluoro-phenyl)-7-hydroxy-4-[3-(2-methyl-imidazol-1-yl)-phenyl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

8-(2-Fluoro-phenyl)-7-hydroxy-4-[3-(2-methylsulfanyl-imidazol-1-yl)-phenyl]-1,3-dihydro-benzo[b][1,4] diazepin-2-one;

8-(2,5-Difluoro-phenyl)-7-methoxy-4-(3-[1,2,3]triazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

8-(2-Fluoro-phenyl)-7-hydroxy-4-[3-(3-methyl-isoxazol-5-yl)-phenyl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one;

3-[7-(2,5-Difluoro-phenyl)-8-hydroxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-benzonitrile;

8-(4-Fluoro-phenylethynyl)-7-hydroxy-4-(3-imidazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one; and

8-(4-Fluoro-phenylethynyl)-7-hydroxy-4-(3-[1,2,3]triazol-1-yl-phenyl)-1,3-dihydro-benzo[b][1,4]diazepin-2-one.

4. The compound according to claim **1**, wherein R^3 is unsubstituted thiophenyl, or thiophenyl substituted by a substituent selected from the group consisting of cyano, halogen, unsubstituted pyridinyl, pyridinyl substituted in 2-position by cyano or halogen, unsubstituted thiazolyl and thiazolyl substituted in 2-position with imidazolyl or 4-methylimidazolyl.

5. The compound according to claim **4**, selected from the group consisting of

5-(7-(2-Fluoro-phenyl)-8-methoxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-thiophene-2-carbonitrile;

2-[7-(2-Fluoro-phenyl)-8-hydroxy-4-oxo-4,5-dihydro-1H-benzo[b]1,4]diazepin-2-yl]-thiophene-3-carbonitrile;

4-[7-(2-Fluoro-phenyl)-8-methoxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2yl]-pyridine-2-carbonitrile;

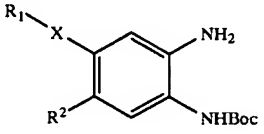
4-[7-(4-Fluoro-phenyl)-8-hydroxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-pyridine-2-carbonitrile;

4-[7-(2-Fluoro-phenyl)-8-hydroxy-4-oxo-4,5-dihydro-3H-benzo[b][1,4]diazepin-2-yl]-pyridine-2-carbonitrile; and

8-(2-Fluoro-phenyl)-4-[2-(4-methyl-imidazol-1-yl)-thiazol-4-yl]-1,3-dihydro-benzo[b][1,4]diazepin-2-one.

6. A pharmaceutical composition comprising at least one compound claimed in claim **1** and pharmaceutically acceptable excipients.

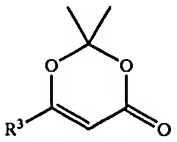
7. A process for preparing a compound according to claim **1**, comprising reacting a compound of formula II:



II

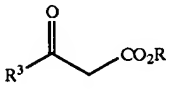
where R^1, R^2 and X are as set forth in claim **1**,

with a compound of formula IV or IVa:



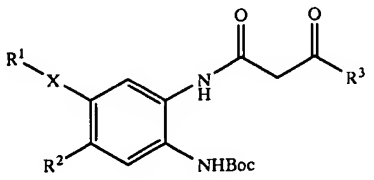
IV

or



IVa

wherein R is ethyl or butyl, and R^3 is as set forth in claim 1, thereby yielding the compound of formula III:



III

subsequently deprotecting the amino group of the compound of formula III; and then cyclizing the deprotected amino compound to obtain a compound of formula I of claim 1.

* * * * *

Ref. 2

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(54) **METABOTROPIC GLUTAMATE RECEPTOR ANTAGONISTS FOR TREATING TOLERANCE AND DEPENDENCY**

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(57) **ABSTRACT**

An antagonist of the metabotropic glutamate receptor 5 (mGluR5) is useful in tolerance or dependence therapy. Such an antagonist can therefore be used in the treatment of substance tolerance or dependence, bulimia nervosa, anorexia nervosa, gambling dependence, sex dependence or obsessive compulsive disorders.

Figure 1a

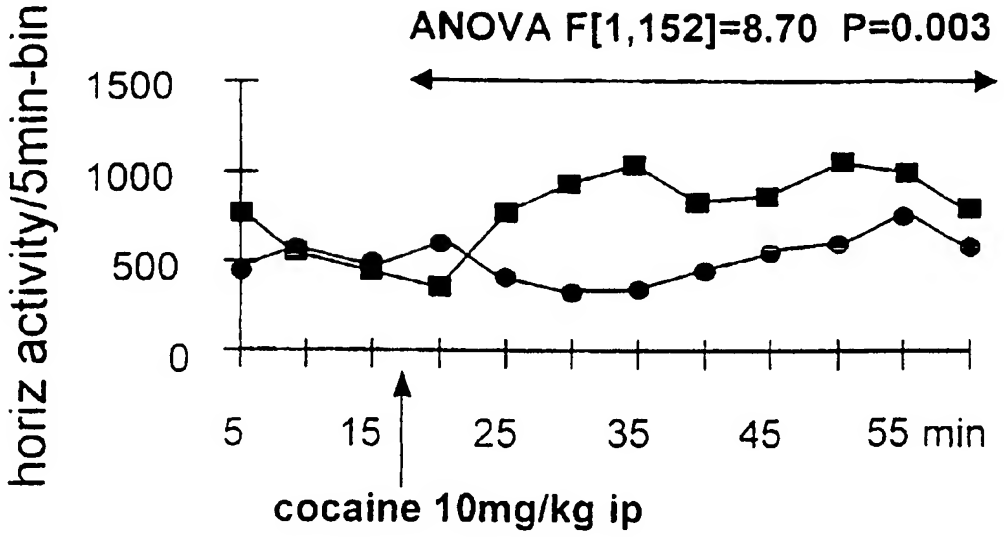


Figure 1b

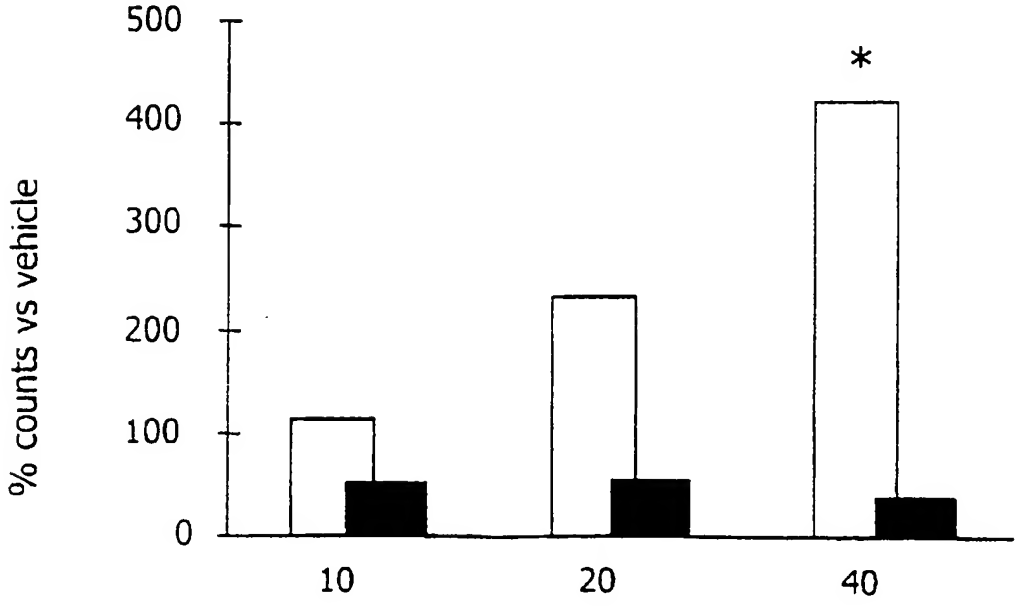


FIGURE 1

Figure 2a

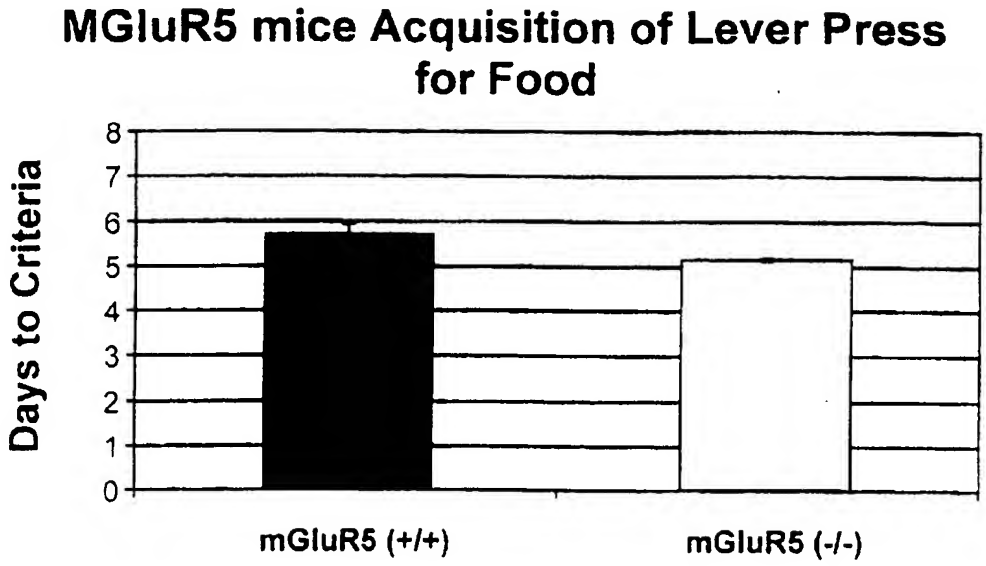


Figure 2b

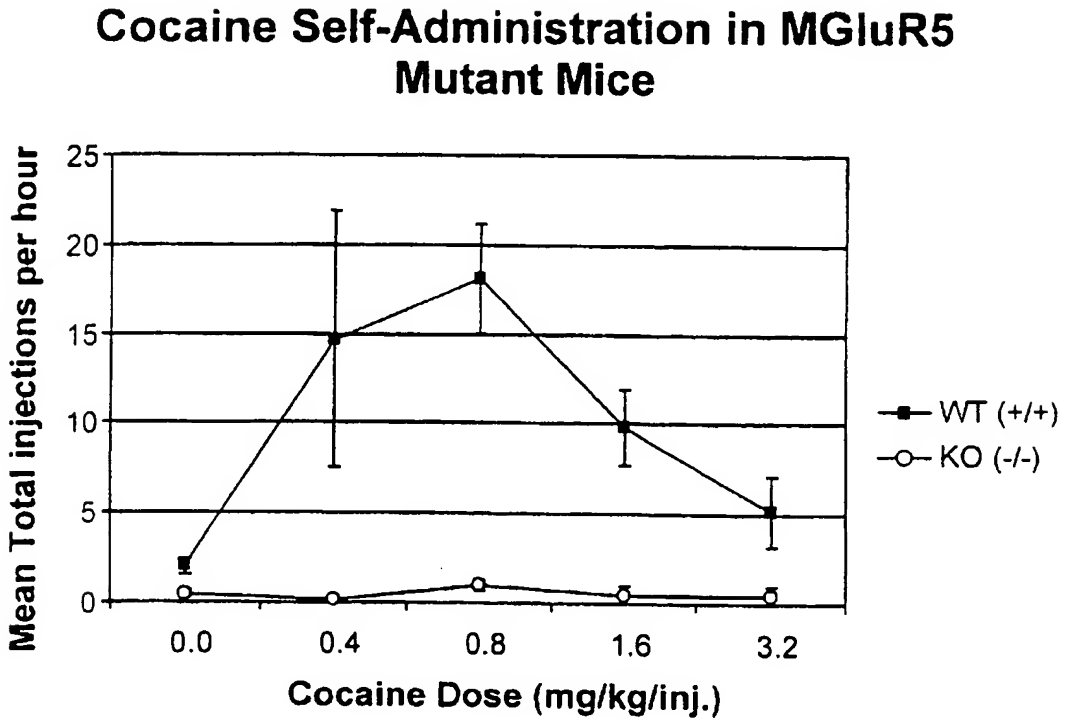


FIGURE 2

Figure 3a
Cocaine effect on NAc DA of mGluR5 +/+

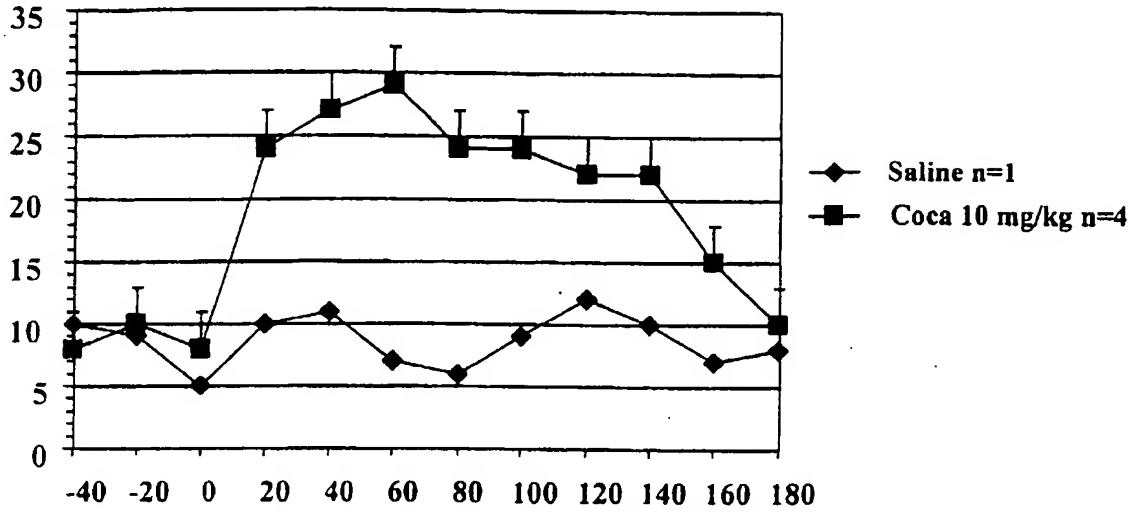


Figure 3b
Cocaine effect on NAc DA of mGluR5 -/-

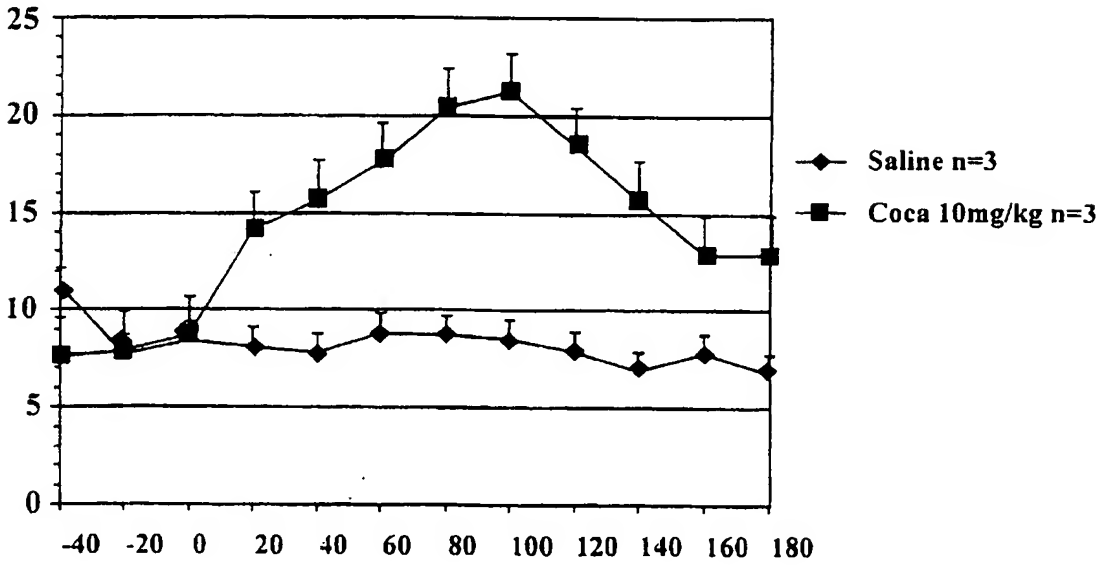


FIGURE 3

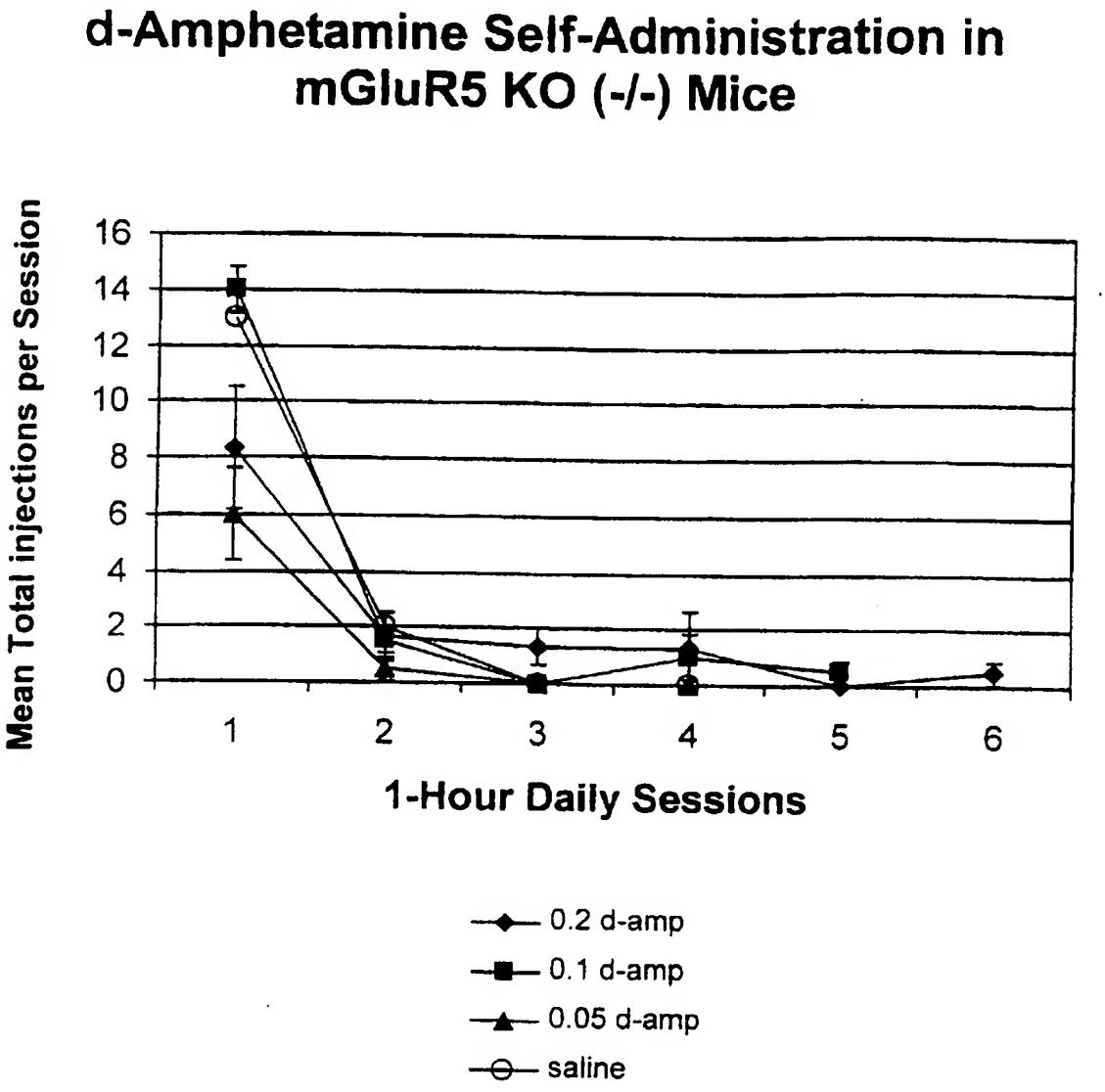


FIGURE 4

METABOTROPIC GLUTAMATE RECEPTOR ANTAGONISTS FOR TREATING TOLERANCE AND DEPENDENCY

FIELD OF THE INVENTION

[0001] The invention relates to tolerance and dependence therapy. It also relates to screening methods for identifying new products which can be used in tolerance and dependence therapy.

BACKGROUND TO THE INVENTION

[0002] Addiction is a chronic brain disease manifested by humans in a variety of behaviours and in a range of social circumstances. Although it is a complex phenomenon, its medical definition is a central nervous system (CNS) disorder manifested as a behavioural disturbance due to a neurobiological imbalance in the brain (Leshner, 1997, Science 278, 45). Individuals may become addicted to a wide variety of factors, including substances like drugs. The obsessive and compulsive aspect of drug dependence may overlap with other obsessive compulsive behaviours such as gambling or compulsive sexual activity.

[0003] In respect of substance abuse, addict behaviour is induced and maintained in a multifactorial fashion with a central role played by the unconditioned reinforcing properties of the abused drug. There are many different substances on which individuals may become dependent, including opiates, benzodiazepines, amphetamine, nicotine, cocaine and ethanol.

[0004] The impact of substance dependence is huge. For example, nicotine dependence is the most widely diffused type of drug addiction. One third of the worldwide population over 15 years of age are smokers. Smoking continues to increase among adolescents and by the year 2025 the WHO estimates that there will be 10 million tobacco related deaths per year. Stopping smoking may evoke a range of symptoms in dependent individuals, including craving, depression, anxiety, difficulty in concentrating and weight gain. Despite a variety of available treatments many smokers fail to give up smoking.

[0005] There is therefore a major unmet need in the area of substance abuse for pharmacological agents that are more effective that those currently available at reducing withdrawal symptoms and more importantly reducing relapse rates. Indeed smoking cessation is a therapeutic area with generally poor results: an avearage 30% success rate compared with 50 to 80% for alcoholism, opioid and cocaine dependence (at 6 months).

[0006] Nevertheless the rationale for pharmacological intervention is, however, still strong because only pharmacotherapy potentially acts on a population larger than that treated with psychosocial interventions and therefore may enhance these traditional methods by improving compliance and quality of the treatment.

[0007] Glutamate is the transmitter of the vast majority of excitatory synapses in the mammalian CNS and plays an important role in a wide variety of CNS functions. In the past, the actions of glutamate in the mammalian brain were thought to be mediated exclusively by activation of glutamate-gated cation channels termed ionotropic glutamate receptors: see Watkins & Evans, Ann. Rev. Pharmacol. Toxicol., 21, 165 (1985). In the mid-1980s, however, evidence for the existence of glutamate receptors directly coupled to a second messenger via G-proteins began to appear with the discovery of a glutamate receptor coupled to activation of phosphonoinositide hydrolysis. That led to the discovery of a new family of glutamate receptors named metabotropic glutamate receptors (mGluRs): see for example. Sladeczek et al. Nature. 317, 717 (1985) and Sugiyama et al., Nature, 325, 531 (1987).

[0008] The search for mGluR-related cDNAs has resulted in the isolation of eight genes that encode distinct mGluRs. These receptors are named mGluR1 through mGluR8. Based on their amino acid sequence identity the eight mGluRs can be classified into three groups. Group I includes mGluR1 and mGluR5, group II mGluR2 and mGlu and group III mGluR4, mGluR6, mGluR7 and mGluR8. Whereas group I mGluRs stimulate inositol phosphate metabolism and mobilization of intracellular Ca^{2+}, both group II and group III mGluRs are negatively coupled to adenylyl cyclase (Schoepp & Conn, Trend Pharmacol. Sci., 14, 13, 1993 and Pin & Duvoisin, Neuropharmacology, 34, 1 (1995)).

SUMMARY OF THE INVENTION

[0009] This invention is based on our findings that:

[0010] (i) a compound which can act as a selective antagonist of the metabotropic glutamate receptor, mGluR5, can reduce the reinstatement of nicotine-seeking behaviour in rats following exposure to experimental determinants of smoking relapse; and

[0011] (ii) mGluR5 knockout mice do not display cocaine-induced hyperactivity and show no response to the reinforcing properties of cocaine and do not self-administer amphetamine at any tested dose.

[0012] We propose that pharmacological block of mGluR5 leads to negative regulation of dopamine-2 (D2) receptors, which in turn reduces dopaminergic activity. The reduction in dopaminergic activity leads to a reduction in smoking relapse. We also propose that mGluR5 is responsible for the hyperactive response to cocaine administration and is also an essential component of the reward process induced by cocaine and amphetamine.

[0013] Furthermore, we propose that mGluR5 is involved in "emotional learning". Addiction implies that an individual has first "learned" how to be dependent before being addicted. Every memorisation process is preceded and we suggest that mGluR5 is responsible for the "learning" process of dependence, regardless of the type of dependence in question. The mGluR5 receptor is thus required for the onset of the dependence process, before the final settlement of physiological addiction.

[0014] According to the present invention there is thus provided use of an antagonist of mGluR5, typically human mGluR5, in the manufacture of a medicament for use in a method of tolerance or dependence therapy.

[0015] The invention also provides:

[0016] an antagonist of mGluR5 for use in a method of treatment of the human or animal body by therapy;

[0017] a method of treating a host suffering from tolerance or dependence, which method comprises administering to the host a therapeutically effective amount of an antagonist of mGluR5;

[0018] a pharmaceutical composition comprising an antagonist of mGluR5 and a pharmaceutically acceptable carrier or diluent;

[0019] products containing an antagonist of mGluR5 and a therapeutic substance as a combined preparation for simultaneous, separate or sequential use in the treatment of a condition for which the said therapeutic substance is used, wherein the use of the therapeutic substance in the absence of said antagonist could lead to tolerance of or dependence on the therapeutic substance;

[0020] use of mGluR5 for identifying a product for use in the treatment tolerance or dependence;

[0021] a method for identifying a product for use in the treatment of tolerance or dependence, comprising:

[0022] (a) contacting a test product with mGluR5 under conditions that in the absence of the test substance would lead to activity of the said mGluR5 and

[0023] (b) determining whether the test product antagonises mGluR5 activity, thereby to determine whether the test product may be used in the treatment of substance tolerance or dependence;

[0024] a product identified by a method of the invention;

[0025] a product of the invention for use in a method of treatment of the human or animal body by therapy;

[0026] use of a product of the invention for the manufacture of a medicament for use in tolerance or dependence therapy;

[0027] a method of treating a host suffering from tolerance or dependence, which method comprises administering to the host a therapeutically effective amount of a product of the invention;

[0028] a pharmaceutical composition comprising a product of the invention and a pharmaceutically acceptable carrier or diluent; and

[0029] products containing a product of the invention and a therapeutic substance as a combined preparation for simultaneous, separate or sequential use in the treatment of a condition for which the said therapeutic substance is used, wherein the use of the therapeutic substance in the absence of said product could lead to tolerance of or dependence on the therapeutic substance.

BRIEF DESCRIPTION OF THE FIGURES

[0030] **FIG. 1(*a*)** shows the effects of cocaine on mutant (●) and wild-type mice (■) mice motor activity. Horizontal activity was measured every 5 minutes during a 60-min session. Mice were injected with saline i.p. and placed in the apparatus at time 0. At time 15 min, mice were injected with cocaine 10 mg/kg i.p. and placed again in the apparatus. Statistics (oneway ANOVA; n=5-12) was performed by comparison of values from time bin 20 to 60 min. SEM are omitted.

[0031] (b) shows the effects of cocaine on mutant (filled bars) and wild-type mice (open bars) motor activity. The total amount of horizontal activity measure during the 60-min session was calculated as percentage of vehicle treatment effect (% counts vs vehicle). Mice were injected with saline i.p. and placed in the apparatus at time 0. At time 15 min. mice were injected with cocaine 10, 20 or 40 mg/kg i.p. and placed again in the apparatus. Statistics (oneway ANOVA followed by Dunnett's; *=P<0.05; n=14-16) were performed by comparison of values from time bin 20 to 60 min. SEM are omitted.

[0032] **FIG. 2** shows the results of cocaine self-administration experiments;

[0033] (a) Learing task experiment with food reinforcer (sugar milk) shows that both mGlu(+/+) (filled bar) and (-/-) (open bar) performed an equal operant behaviour; and

[0034] (b) Dose response curve at different doses of cocaine in self-administration paradigm for wild type (■) and knockout (O)mice.

[0035] **FIG. 3** shows the results of dopamine microdialysis in the nucleus accumbens of (a) wild type and (b) knockout mice after injection of 10 mg/kg of cocaine or saline buffer intraperiteonally.

[0036] **FIG. 4** shows d-amphetamine self-administration in mGluR5 knock-out (-/-) mice.

DETAILED DESCRIPTION OF THE INVENTION

[0037] The present invention is concerned with antagonists of mGluR5 for treating tolerance or dependence. The mGluR5 is preferably human mGluR5.

[0038] An antagonist of mGluR5 is a substance which diminishes or abolishes the effect of a ligand (agonist) which typically activates mGluR5. Thus, the antagonist may be, for example, a chemical antagonist, a pharmacokinetic antagonist, an antagonist by receptor block, a non-competitive antagonist or a physiological antagonist.

[0039] A chemical antagonist is wherein the antagonist binds the ligand in solution so the effect of the ligand is lost.

[0040] A pharmacokinetic antagonist is one which effectively reduces the concentration of the active drug at its site of action, for example by increasing the rate of metabolic degradation of the active ligand.

[0041] Antagonism by receptor-block involves two important mechanisms: reversible competitive antagonism and irreversible, or non-equilibrium, competitive antagonism. Reversible competitive antagonism occurs when the rate of dissociation of the antagonist molecules is sufficiently high that, on addition of the ligand, displacement of the antagonist molecules from the receptors effectively occurs. Of course the ligand cannot evict a bound antagonist molecule, or vice versa. Irreversible or non-equilibrium, competitive antagonism occurs when the antagonist dissociates very slowly, or not at all, from the receptor with the result that no change in the antagonist occupancy takes place when the ligand is applied. Thus the antagonism is insurmountable.

[0042] Non-competitive antagonism describes the situation where the antagonist blocks at some point in the signal transduction pathway leading to the production of a response by the ligand.

[0043] Physiological antagonism is a term used loosely to describe the interaction of two substances whose opposing actions in the body tend to cancel each other out.

[0044] An antagonist can also be a substance which diminishes or abolishes expression of functional mGluR5. Thus an antagonist can be, for example, a substance which diminishes or abolishes expression of the gene encoding mGluR5, diminishes or abolishes translation of mGluR5 RNA, diminishes or abolishes post-translational modification of mGluR3 protein or diminishes or abolishes the insertion of mGluR5 into the cell membrane.

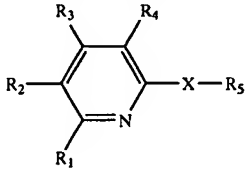
[0045] Preferred antagonists are those which lead to a reduction of activation by the ligand of at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99% at a concentration of the antagonist of 1 μgml^{-1}, 10 μgml^{-1}, 100 μgml^{-1}, 500 μgml^{-1}, 1 $mgml^{-1}$, 10 $mgml^{-1}$, 100 mg ml^{-1}. The percentage antagonism represents the percentage decrease in activity of mGluR5 in a comparison of assays in the presence and absence of the antagonist. Any combination of the above mentioned degrees of percentage antagonism and concentration of antagonist may be used to define an antagonist of the invention, with greater antagonism at lower concentrations being preferred.

[0046] An antagonist for use in the invention may be a relatively non-specific antagonist which is an antagonist of mGluRs in general. Preferably, however, an antagonist antagonises only group I mGluRs. More preferably, an antagonist used in the invention is a selective antagonist of mGluR5. A selective antagonist of mGluR5 is one which antagonises mGluR5, but antagonises other mGluRs only weakly or substantially not at all. Most preferred antagonists are those which can selectively antagonise mGluR5 at low concentrations, for example those that cause a level of antagonism of 50% or greater at a concentration of 100 μgml^{-1} or less.

[0047] Selective mGluR5 antagonists can thus typically exhibit at least 100 fold greater activity at an mGluR5 receptor than at an mGluR1 receptor, preferably at least 200 fold greater activity and most preferably at least 400 fold greater activity. They can display a high degree of selectivity and affinity as antagonists of the human and/or rat mGluR5.

[0048] Suitable antagonists for use in the invention are disclosed in EP-A-0807621, WO 99/02497, WO 00/20001 and WO 00/63166. As disclosed in WO 99/02497, therefore, suitable antagonists may thus have the formula (I):

(I)



[0049] wherein

[0050] R_1 denotes hydrogen, lower alkyl, hydroxy-lower alkyl, lower alkyl-amino, piperidino, carboxy, esterified carboxy, amidated carboxy, unsubstituted or lower alkyl-, lower alkoxy-, halo- and/or trifluoromethyl-substituted N-lower-alkyl-CN-phenylcarbamoyl, lower alkoxy, halo-lower alkyl or halo-lower alkoxy;

[0051] R_2 denotes hydrogen, lower alkyl, carboxy, esterified carboxy, amidated carboxy, hydroxy-lower alkyl, hydroxy, lower alkoxy or lower alkanoyloxy, 4-(4-fluoro-benzoyl)-piperidin-1-ylcarboxy, 4-t.butyloxycarbonyl-piperazin-1-yl-carboxy, 4-(4-azido-2-hydroxybenzoyl)-piperazin-1-yl-carboxy or 4-(4-azido-2-hydroxy-3-iodo-benzoyl)-piperazin-1-yl-carboxy;

[0052] R_3 represents hydrogen, lower alkyl, carboxy, lower alkoxy-carbonyl, lower alkyl-carbamoyl, hydroxy-lower alkyl, di-lower alkyl-aminomethyl, morpholinocarbonyl or 4-(4-fluoro-benzoyl)-piperadin-1-yl-carboxy;

[0053] R_4 represents hydrogen, lower alkyl, hydroxy, hydroxy-lower alkyl, amino-lower alkyl, lower alkylamino-lower alkyl, di-lower alkylamino-lower alkyl, unsubstituted or hydroxy-substituted lower alkyleneamino-lower alkyl, lower alkoxy, lower alkanoyloxy, amino-lower alkoxy, lower alkylamino-lower alkoxy, di-lower alkylamino-lower alkoxy, phthalimido-lower alkoxy, unsubstituted or hydroxy- or 2-oxo-imidazolidin-1-yl-substituted lower alkyleneamino-lower alkoxy, carboxy, esterified or amidated carboxy, carboxy-lower alkoxy or esterified carboxy-lower alkoxy;

[0054] X represents an optionally halo-substituted lower alkenylene or alkynylene group bonded via vicinal saturated carbon atoms or an azo (—N=N—) group, and R_5 denotes an aromatic or heteroaromatic group which is unsubstituted or substituted by one or more substituents selected from lower alkyl, halo, halo-lower alkyl, halo-lower alkoxy, lower alkenyl, lower alkynyl, unsubstituted or lower alkyl-, lower alkoxy-, halo- and/or trifluoromethyl-substituted phenyl, unsubstituted or lower alkyl-, lower alkoxy-, halo and/or trifluoromethyl-substituted phenyl-lower alkynyl, hydroxy, hydroxy-lower alkyl, lower alkanoyloxy-lower alkyl, lower alkoxy, lower alkenyloxy, lower alkylenedioxy, lower alkanoyloxy, amino-, lower alkylamino-, lower alkanoylamino- or N-lower alkyl-N-lower alkanoylamino-lower alkoxy, unsubstituted or lower alkyl-, lower alkoxy-, halo- and/or trifluoromethyl-substituted phenoxy, unsubstituted or lower alkyl-, lower alkoxy-, halo and/or trifluoromethyl-substituted phenyl-lower alkoxy, acyl, carboxy, esterified carboxy, amidated carboxy, cyano, carboxy-lower alkylamino, esterified carboxy-lower alkylamino, amidated carboxy-lower alkylamino, phosphono-lower alkylamino, esterified phosphono-lower alkylamino, nitro, amino, lower alkylamino, di-lower alkylamino-acylamino, N-acyl-N-lower alkylamino, phenylamino, phenyl-lower alkylaamino, cycloalkyl-lower alkylamino or heteroaryl-lower alkylamino each of which may be unsubstituted or lower alkyl-, lower alkoxy-, halo- and/or trifluoromethyl-substituted; their N-oxides and their pharmaceutically acceptable salts.

[0055] Compounds of formula (I) which have basic groups may form acid addition salts and compounds of the formula

(I) having acidic groups may form salts with bases. Compounds of formula (I) having basic groups and in addition having at least one acidic group, may also form internal salts. Also included are both total and partial salts, that is to say salts with 1, 2 or 3, preferably 2, equivalents of base per mole of acid of formula (I), or salts with 1, 2 or 3 equivalents, preferably 1 equivalent, of acid per mole of base of formula (I). Only the pharmaceutically acceptable, non-toxic salts are used therapeutically and they are therefore preferred.

[0056] Halo in the present description denotes fluorine, chlorine, bromine or iodine. Lower alkyl is typically C_{1-6}, for example C_{1-4}, alkyl. Lower alkoxy is typically C_{1-6}, for example C_{1-4}, alkoxy. Lower alkynyl is typically C_{2-5} alkynyl. Lower alkanoyl is typically C_{2-5}alkanoyl. Lower alkylene is typically C_{2-5} alkylene. Lower alkenylene is typically C_{2-5} alkenylene. Lower alkynylene is typically C_{2-4} alkynylene.

[0057] When X represents an alkenylene group, configuration trans is preferred.

[0058] Preferred compounds of formula (I) are those wherein:

[0059] X represents an optionally halo-substituted (C_{2-4})alkenylene or alkynylene group bonded via vicinal unsaturated carbon atoms.

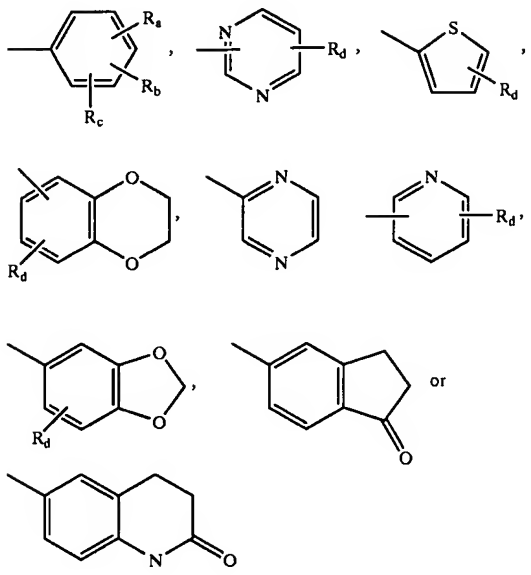
[0060] R_1 is hydrogen, (C_{1-4}) alkyl, (C_{1-4})alkoxy, hydroxy(C_{1-4})alkyl, cyano, ethynyl, carboxy, (C_{1-4})alkoxycarbonyl, di(C_{1-4})alkylamino, (C_{1-6})alkylaminocarbonyl, trifluoromethylphenylaminocarbonyl,

[0061] R_2 is hydrogen hydroxy (C_{1-4})alkyl, hydroxy (C_{1-4})alkyl, (C_{1-4})alkoxy, carboxy, (C_{2-5})alkanoyloxy, (C_{1-4})alkoxycarbonyl, di(C_{1-4})alkylamino(C_{1-4})alkanoyl, -di(C_{1-4})alkylaminomethyl, 4-(4-fluorobenzoyl)-piperidin-1-yl-carboxy, 4-t.-butyloxycarbonyl-piperazin-1-yl-carboxy, 4-(4-azido-2-hydroxybenzoyl)-piperazin-1-yl-carboxy or 4-(4-azido-2-hydroxy-3-iodo-benzoyl)-piperazin-1-yl-carboxy,

[0062] R_3 is hydrogen, (C_{1-4}) alkyl, carboxy, (C_{1-4})alkoxycarbonyl, (C_{1-4})alkyl-carbamoyl, hydroxy(C_{1-4})alkyl, di(C_{1-4})alkylaminomethyl, morpholinocarbonyl or 4-(4-fluoro-benzoyl)-piperidin-1-yl-carboxy,

[0063] R_4 is hydrogen, hydroxy, (C_{1-4})alkoxy, carboxy, (C_{2-5})alkanoyloxy, (C_{1-4})alkoxy-carbonyl, amino(C_{1-4})alkoxy, di(C_{1-4})alkylamino(C_{1-4})alkoxy, di(C_{1-4})alkylamino(C_{1-4})alkyl, carboxy (C_{1-4})alkylcarbonyl, (C_{1-4})alkoxycarbonyl(C_{1-4})alkoxy, hydroxy(C_{1-4})alkyl, di(C_{1-4})alkylamino(C_{1-4})alkoxy, m-hydroxy-p-azidophenylcarbonylamino(C_{1-4})alkoxy, and

[0064] R_5 is a group of formula



[0065] wherein:

[0066] R_a and R_b independently are hydrogen, hydroxy, halogen, nitro, cyano, carboxy, (C_{1-4})alkyl (C_{1-4})alkoxy, hydroxy(C_{1-4})alkyl, (C_{1-4})alkoxycarbonyl, (C_{2-7})alkanoyl, (C_{2-5})alkanoyloxy, (C_{2-5})alkanoyloxy(C_{1-4})alkyl, trifluoromethyl, trifluoromethoxy, trimethylsilylethynyl, (C_{2-5})alkynyl, amino, azido, amino(C_{1-4})alkoxy, (C_{2-5})alkanoylamino(C_{1-4})alkoxy, (C_{1-4})alkylamino(C_{1-4})alkoxy, di(C_{1-4})alkylamino(C_{1-4})alkoxy, (C_{1-4})alkylamino, di(C_{1-4})alkylamino, monohalobenzylamino, thienylmethylamino, thienylcarbonylamino, trifluoromethylphenylaminocarbonyl, tetrazolyl, (C_{2-5})alkanoymamino, benzylcarbonylamino, (C_{1-4})alkylaminocarbonylamino. (C_{1-4})alkoxycarbonylaminocarbonylamino or (C_{1-4})alkylsulfonyl,

[0067] R_c is hydrogen, fluorine, chlorine, bromine, hydroxy, (C_{1-4})alkyl, (C_{2-5})alkanoyloxy, (C_{1-4})alkoxy or cyano, and

[0068] R_d is hydrogen, halogen or (C_{1-4})alkyl.

[0069] More preferred compounds of formula (I) are those wherein X is as defined above and

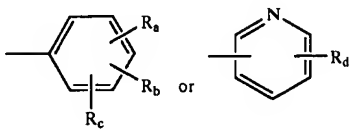
[0070] R_1 is hydrogen, (C_{1-4})alkyl, (C_{1-4})alkoxy, cyano, ethynyl or di(C_{1-4})alkylamino,

[0071] R_2 is hydrogen, hydroxy carboxy, (C_{1-4}) alkoxycarbonyl, di(C_{1-4})alkylaminomethyl, 4-(4-fluoro-benzoyl)-piperdin-1-yl-carboxy-4-t.-butyloxycarbonyl-piperizin-1-ylcarboxy, 4-(4-azido-2-hydroxybenzoyl)-pipeazin-1-yl-carboxy or 4-(4-azido-2-hydroxy-3-iodo-benzoyl)-piperazin-1-yl-carboxy,

[0072] R_3 is as defined above,

[0073] R_4 is hydrogen, hydroxy, carboxy, (C_{2-5})alkanoyloxy, (C_{1-4})alkoxycarbonyl, amino(C_{1-4})alkoxy, di(C_{1-4})alkylamino(C_{1-4})alkoxy, di(C_{1-4})alkylamino(C_{1-4})alkyl or hydroxy(C_{1-4})alkyl, and

[0074] R_5 is a group of formula

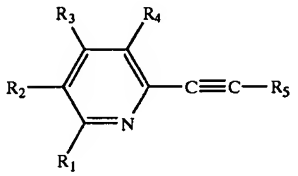


[0075] wherein:

[0076] R_a and R_b independently are hydrogen, halogen, nitro, cyano, $(C_1,)$alkyl, $C_{(1-4}$)alkoxy, trifluoromethyl, trifluoromethoxy or (C_{2-5})alkynyl, and R and Rd are as defined above.

[0077] Further selective mGluR antagonists are 2-arylalkenyl-, 2-heteroarylalkenyl-, 2-arylalkynyl-, 2-heteroarylalkynyl, 9-arylazo- and 2-heteroarylazo-pyridines, more particularly 6-methyl-2-(phenylazo)-3'-pyridinol, (E)-2-methyl-6-styryl-pyridine and -(phenylazo)-3-pyridinol, (E)-2-methyl-6-styryl-pyridine and compounds of formula (II):

(II)



[0078] wherein

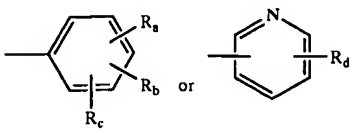
[0079] R_1 is hydrogen, (C_{1-4})alkyl, (C_{1-4})alkoxy, cyano, ethynyl or di(C_{1-4})alkylamino,

[0080] R_2 is hydrogen, hydroxy, carboxy, (C_{1-4}) alkoxycarbonyl, di(C_{1-4})alkylaminomethyl, 4-(4-fluoro-benzoyl)-piperidin-1-yl-carboxy, 4-t.butyloxycarbonyl-piperazin-1-yl-carboxy, 4-(4-azido-2-hydroxybenzoyl)-piperazin-1-yl-carboxy or 4-(4-azido-2-hydroxy-3-iodo-benzoyl)-piperazin-1-yl-carboxy,

[0081] R_3 is hydrogen, (C_{1-4})alkyl, carboxy, (C_{1-4})alkoxycarbonyl, (C_{1-4})alkylcarbamoyl, hydroxy(C_{1-4})alkyl, di(C_{1-4})alkylaminomethyl, morpholinocarbonyl or 4-(4-fluoro-benzoyl)-piperazin-1-yl-carboxy,

[0082] R_4 is hydrogen, hydroxy, carboxy, $C_{(2-5)}$alkanoyloxy, (C_{1-4})alkoxycarbonyl, amino (C_{1-4})alkoxy, di(C_{1-4})alkylamino$(C_{,4})$alkoxy, di(C_{1-4})alkylamino(C_{1-4})alkyl or hydroxy(C_{1-4})alkyl, and

[0083] R_5 is a group of formula



[0084] wherein

[0085] R_a and R_b independently are hydrogen, halogen, nitro, cyano, (C_{1-4})alkyl, (C_{1-4})alkoxy, trifluoromethyl, trifluoromethoxy or (C2,)alkynyl, and

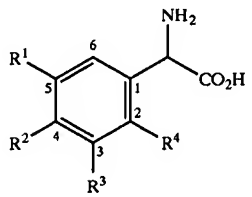
[0086] R_c is hydrogen, fluorine, chlorine bromine, hydroxy (C_{1-4})alkyl, $C_{(2-5)}$alkanoyloxy, (C_{1-4})alkoxy or cyano; and

[0087] R_d is hydrogen, halogen or (C_{1-4})alkyl;

[0088] in free form or in form of pharmaceutically acceptable salts.

[0089] Suitable phenyl glycine compounds are disclosed in EP-A-0807621. Phenyl glycine compounds useful in the invention can thus have the formula (III):

(III)



[0090] in which R^1 is hydrogen, hydroxy or C_{1-6} alkoxy;

[0091] R_2 is hydrogen, carboxy, tetrazolyl, —SO_2H, —SO_3H, —OSO_3H, —CONHOH, or —P(OH)OR', —PO(OH)OR', —OP(OH)OR' or —OPO(OH)OR' where R' is hydrogen, C_{1-6} alkyl, C_{2-6} alkenyl or aryl C_{1-6} aryl;

[0092] R_3 is hydrogen, hydroxy or C_{1-4} alkoxy; and

[0093] R_4 is fluoro, trifluoromethyl, nitro, C_{1-6} alkyl, C_{3-7} cycloalkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{1-6} alkylthio, heteroaryl, optionally substituted aryl, optionally substituted aryl C_{1-6} alkyl, optionally substituted aryl C_{2-6} alkenyl, optionally substituted aryl C_{2-6} alkynyl, optionally substituted aryloxy, optionally substituted C_{1-6} alkoxy, optionally substituted arylthio, optionally substituted aryl C_{1-4} alylthio or —CONR"R'", —NR"R'", —OCONR"R'" or —SONR"R'" where R" and R'" are each hydrogen, C_{1-6} alkyl or aryl C_{1-6} alkyl, or R" and R'" together form a C_{3-7} alkylene ring;

[0094] or a salt or ester thereof:

[0095] In an embodiment, R^1, R^2 and R^3 are not all hydrogen. In another embodiment, R^4 is not fluoro when R^2 and R^5 are hydrogen and R^1 is hydroxy.

[0096] In the above formulae and alkyl group can be straight or branched chain, such as, for example, methyl, ethyl, propyl, isopropyl, butyl and isobutyl, and is preferably methyl or ethyl. A C_{2-6} alkenyl group includes, for example, vinyl, prop-2-enyl, but-3-enyl, pent-4-enyl and isopropenyl. A preferred alkenyl group is of the formula R—CH=CH— where R is C_{1-4} alkyl. An alkynyl group includes, for example, prop-2-ynyl, but-3-ynyl, and pent-t-ynyl. A preferred alkynyl group is of the formula:

R—C≡C—

[0097] where R is C_{1-4} alkyl. A C_{3-7} cycloalkyl group is preferably, for example, cyclopropyl, cyclopentyl or cyclohexyl and these groups may optionally be substituted by one or two methyl substituents.

[0098] An aryl group is preferably phenyl or naphthyl, and an optionally substituted phenyl or naphthyl group is optionally substituted with, for example, one or more substituents, preferably 1 to 3 substituents, selected from C_{1-4} alkyl, especially methyl, C_{1-4} alkoxy, especially methoxy and ethoxy, carboxy, hydroxy, cyano, halo, especially bromo, chloro and fluoro, trifluoromethyl, nitro, amino, C_{1-4} acylamino and C_{1-4} alkylthio. A napthyl group can be 1-naphthyl or 2-naphtyl. When substituted, a phenyl or naphthyl group is preferably substituted by one to three substituents. An aryl C_{1-6} alkyl group is one such group linked through an alkylene chain, for example, aryl $(CH_2)_n$ where n is 1 to 6, and a most preferred example is benzyl. Preferred examples of groups as are follows:

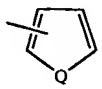
[0099] aryloxy—optionally substituted phenoxy:

[0100] aryl C_{1-6} alkoxy—optionally substituted phenylmethyoxy or phenylethoxy;

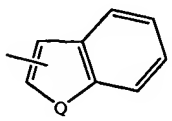
[0101] arylthio—optionally substituted phenylthio;

[0102] aryl C_{1-6} alkylthio—optionally substituted phenylmethythio or phenylethylthio.

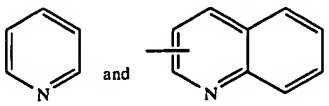
[0103] A heteroaryl group can be aryl group having one or more hetero atoms in the ring. The term includes fused ring structures. Preferably the heteroaryl group contains one or two hetero atoms selected from oxygen, nitrogen and sulphur. It preferably contains from 5 to 10 carbon atoms, and for example may be of the formula:



[0104] where Q is —O, —S— or —NR—, and R is hydrogen or C_{1-4} alkyl. Alternatively, a heteroaryl group comprises a benzene fused ring as, for example:



[0105] and further heteroaryl groups include:



[0106] Especially preferred heteroaryl groups are pyrrolyl, thieneyl or furanyl, preferred examples being 2-thieneyl and 2-furanyl, and also pyridyl, in particular 2- and 3-pyridyl.

[0107] The group R^2 is preferably hydrogen, carboxy or tetrazolyl and especially carboxy, and the group R^4 is preferably C_{1-6} alkyl, C_{2-6} alkenyl, optionally substituted phenyl, optionally substituted phenyl C_{1-6} alkyl, optionally substituted phenoxy, optionally substituted phenylithio or optionally substituted phenyl C_{1-6} alklthio.

[0108] The groups R^1 and R^3 are each preferably hydrogen or hydroxy.

[0109] Examples of particular antagonists of mGluR5 that are useful in the invention include 2-methyl-6-(phenylethynyl)-pyridine (MPEP), 2-methyl-6-[(1E)-2 phenylethenyl] pyridine. 6-methyl-(phenylazo)-3-pyridinol, (RS)-α-methyl-4-carboxyphenylglycine (MCPG), and analogues and derivatives thereof.

[0110] An antagonist of mGluR5 may be used in a method of treatment of the human or animal body. The treatment may be a prophylactic treatment. I particular such antagonists may be used in the tolerance and/or dependence therapy. Antagonists may also be used in the manufacture of a medicament for use in tolerance and/or dependence therapy. The condition of a patient suffering from tolerance and/or dependence can be improved by administration of an antagonist of mGluR5. A therapeutically effective amount of an antagonist of mGluR5 may be given to a human patient in need thereof.

[0111] Tolerance and dependence may be treated according to the invention. Tolerance and dependence are separate phenomena.

[0112] Tolerance describes an increase in dose needed to produce a given pharmacological effect of a particular substance. In the case of opiates, for example, tolerance develops rapidly.

[0113] Dependence involves two separate components, namely physical and psychological dependence. Physical dependence is characterised by a clear-cut abstinence syndrome, such that abrupt withdrawal of a substance (cessation) may lead to, for example, increased irritability or body shakes. The exact nature of the abstinence syndrome is related to the particular substance in question. The invention may be used in the treatment of abstinence syndrome/cessation.

[0114] Psychological dependence is more complex than physical dependence and probably more important in the genesis of compulsive substance taking (ie. addiction). Typically, opiate addicts who recover fully from the abstinence syndrome are likely to revert to drug taking later. In animal models of psychological dependence on opiates based on measurement of the potentiality of drugs to act as reinforcers in tests of operant conditioning, the reinforcing effect of the drug outlasts the duration of the physical abstinence syndrome. The invention may used in the treatment of psychological dependence and in the reduction or abolition of the reinforcing effects of drugs.

[0115] The invention is applicable to the treatment of many different forms of tolerance or dependence. Tolerance or dependence may be reduced or abolished. Typically, the invention is applicable to the treatment of substance tolerance or substance dependence.

[0116] In the context of substance tolerance and dependence, the invention is applicable both to what may be

loosely termed "abuse" such as nicotine addiction in the case of smokers or the consumption of other recreational drugs, and to therapeutic usage. For example, therapeutic usage of benzodiazepines and opiates may lead to tolerance to and/or dependence on those drugs. It is clearly advantageous that those consequences of pharmacological therapy effects be ameliorated or abolished.

[0117] Thus, an antagonist of a mGluR5 may be used to prevent addiction to a therapeutic pharmaceutical. In such an application, the antagonist of mGluR5 is administered before the said therapeutic pharmaceutical has itself been administered, after the said pharmaceutical has been administered or after withdrawal of the pharmaceutical, or it may be co-administered with the said pharmaceutical.

[0118] The invention is also of relevance to the treatment of a wide spectrum of other addiction-related conditions. For example, an antagonist of mGluR5 may be used in the treatment of bulimia nervosa, anorexia nervosa, betting and gambling dependence, sex dependence, sporting activity dependence or obsessive compulsive disorder. An antagonist of mGluR5 may thus be used to treat obsessive and/or compulsive behaviours and the obsessive and/or compulsive components of a variety of disorders such as gambling and/or compulsive sexual activity.

[0119] The invention provides treatment of tolerance to and dependence on a number of substances. The invention is particularly useful in the treatment of dependence on nicotine, for example to treat withdrawal effects of smoking cessation. Thus, the invention may be used in the treatment of smoking cessation-related phenomena including craving, depression, anxiety, concentration difficulty and weight gain. Withdrawal symptoms associated with smoking cessation can be reduced.

[0120] The invention is also useful in the treatment of cocaine, amphetamine and alcohol addiction. Dependence on cocaine, amphetamines and alcohol, may be reduced or abolished. Tolerance of and dependence on amphetamine-related drugs such as dextroamphetamine, methylamphetamine, methylphenidate and fenfluramine may also be treated using an antagonist of mGluR5.

[0121] The invention is further useful in the treatment of opiate tolerance or dependence, in both "abuse" contexts, for example dependence on heroin and pharmaceutical contexts, for example in the prophylaxis of morphine tolerance and/or dependence. Additionally, tolerance and dependence on benzodiazepines, including diazepam and temazepam may be treated by the use of an antagonist of mGluR5.

[0122] Antagonists of mGluR5 may be administered in a variety of dosage forms. Thus, they can be administered orally, for example as tablets, troches, lozenges, aqueous or oily suspensions dispersible powders or granules. The antagonists may also be administered parenterally, either subcutaneously, intravenously, intramuscularly, intrasternally, transdermally or by infusion techniques. The inhibitors may also be administered as suppositories. A physician will be able to determine the required route of administration for each particular patient.

[0123] The formulation of an antagonist of mGluR5 will depend upon factors such as the nature of the exact antagonist, whether a pharmaceutical or veterinary use is intended, etc. An antagonist of mGluR5 may be formulated for simultaneous, separate or sequential use.

[0124] An antagonist of mGluR5 is typically formulated for administration in the present invention with a pharmaceutically acceptable carrier or diluent. The pharmaceutical carrier or diluent may be, for example, an isotonic solution. For example, solid oral forms may contain, together with the active compound, diluents, e.g. lactose, dextrose, saccharose, cellulose, corn starch or potato starch; lubricants, e.g. silica, talc, stearic acid, magnesium or calcium stearate, and/or polyethylene glycols; binding agents; e.g. starches, gum arabic, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone; disaggregating agents, e.g. starch, alginic acid, alginates or sodium starch glycolate, effervescing mixtures; dyestuffs; sweeteners; wetting agents, such as lecithin, polysorbates, laurylsulphates; and, in general, non-toxic and pharmacologically inactive substances used in pharmaceutical formulations. Such pharmaceutical preparations may be manufactured in known manner, for example, by means of mixing, granulating, tabletting, sugar-coating, or film-coating processes.

[0125] Liquid dispersions for oral administration may be syrups, emulsions or suspensions. The syrups may contain as carriers, for example, saccharose or saccharose with glycerine and/or mannitol and/or sorbitol.

[0126] Suspensions and emulsions may contain as carrier, for example a natural gum, agar, sodium alginate, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. The suspensions or solutions for intramuscular injections may contain, together with the active compound, a pharmaceutically acceptable carrier, e.g. sterile water, olive oil, ethyl oleate, glycols, e.g. propylene glycol, and if desired, a suitable amount of lidocaine hydrochloride.

[0127] Solutions for intravenous administration or infusion may contain as carrier, for example, sterile water or preferably they may be in the form of sterile, aqueous, isotonic saline solutions.

[0128] A therapeutically effective amount of an antagonist of mGluR5 is administered to a patient. The dose of an antagonist of mGluR5 may be determined according to various parameters, especially according to the substance used; the age, weight and condition of the patient to be treated; the route of administration; and the required regimen. Again, a physician will be able to determine the required route of administration and dosage for any particular patient. A typical daily dose is from about 0.1 to 50 mg per kg of body weight, according to the activity of the specific inhibitor, the age, weight and conditions of the subject to be treated, the type and severity of the degeneration and the frequency and route of administration. Preferably, daily dosage levels are from 5 mg to 2 g.

[0129] The invention also provides methods for identifying products which may be used in a method of treatment of the human or animal body by therapy, in particular in the treatment of tolerance or dependence. Such methods essentially comprise determining whether a test product is a mGluR5 antagonist and determining whether an antagonist so-identified can be used in the treatment of substance tolerance or dependence.

[0130] Antagonists of mGluR5 are defined above and any suitable assay may be used may be carried out in order to

determine whether a test product is an mGluR5 antagonist. Preferably, any assay used will be suitable for high throughput

1. (CANCEL)
2. (CANCEL)
3. (CANCEL)
4. (CANCEL)
5. (CANCEL)
6. (CANCEL)
7. (CANCEL)

8. (amended) A method or treating a mammalian subject suffering from tolerance or dependence, which method comprises administering to the subject a therapeutically effective amount of an antagonist of mGluR5.

9. (amended) A pharmaceutical composition comprising an antagonist of mGluR5and a therapeutic substance, wherein the use of the therapeutic substance in the absence of said antagonist could lead to tolerance of or dependence on the therapeutic substance.

10. (CANCEL)

11. (amended) A method for screening a compound for use in the treatment of tolerance or dependence, comprising:

(a) contacting a test compound with mGluR5 under the conditions that in the absence of the test compound would lead to activity of the said mGluR5; and

determining whether the test compound antagonises mGluR5 activity.

12. (CANCEL)
13. (CANCEL)
14. (CANCEL)
15. (CANCEL)
16. (CANCEL)
17. (CANCEL)

18. (new) A method of treating a subject with abstinence syndrome due to a substance selected from nicotine, an opiate, cocaine, an amphetamine, a benzodiazipine and ethanol,

comprising administering a therapeutically effective amount of an antagonist of mGluR5 to said subject.

19. (new) A method of treating a subject with abstinence syndrome due to a substance selected from an opiate, cocaine, an amphetamine, and a benzodiazipine,

comprising administering a therapeutically effective amount or an antagonist of mGluR5 to said subject.

20. (new) A method of treating a subject with abstinence syndrome due to ethanol,

comprising administering a therapeutically effective amount of an antagonist of mGluR5 to said subject.

21. (new) A method of treating substance dependence in a mammalian subject in need thereof, where said substance is nicotine,

comprising administering a therapeutically effective amount of of an antagonist of mGluR5 to said subject.

22. (new) A method of treating substance dependence in a mammalian subject in need thereof, where said substance is selected from an opiate, cocaine, an amphetamine, and a benzodiazipine,

comprising administering a therapeutically effective amount of of an antagonist of mGluR5 to said subject.

23. (new) A method of treating substance dependence in a mammalian subject in need thereof, where said substance is ethanol,

comprising administering a therapeutically effective amount of of an antagonist of mGluR5 to said subject.

24. (new) A method of treating tolerance to a therapeutic compound, where said compound is selected from opiates, amphetamines, and benzodiazipines,

comprising administering a therapeutically effective amount of of an antagonist of mGluR5 to said subject

25. (new) A method of treating, in a mammalian subject, a condition selected from bulimia nervosa and anorexia, comprising administering a therapeutically effective amount of an antagonist of mGluR5 to said subject.

26. (new) A method of treating, in a mammalian subject, a condition selected from gambling dependence, sex dependence and obsessive compulsive disorder, comprising administering a therapeutically effective amount of an antagonist of mGluR5 to said subject.

27. (new) A pharmaceutical composition comprising pharmaceutically acceptable carrier, an antagonist of mGluR5, and a therapeutic substance selected from opiates, amphetamines, and benzodiazipines.

* * * * *

Ref. 3 (for appear brief USSN 10/527,525)

Reinforcing and locomotor stimulant effects of cocaine are absent in mGluR5 null mutant mice

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Both ionotropic and metabotropic glutamate receptors (mGluRs) are involved in the behavioral effects of pyschostimulants[1–3]; however, the specific contributions of individual mGluR subtypes remain unknown. Here we show that mice lacking the mGluR5 gene do not self-administer cocaine, and show no increased locomotor activity following cocaine treatment, despite showing cocaine-induced increases in nucleus accumbens (NAcc) dopamine (DA) levels similar to wild-type (WT) mice. These results demonstrate a significant contribution of mGlu5 receptors to the behavioral effects of cocaine, and suggest that they may be involved in cocaine addiction.

Both acute and repeated cocaine administration increase glutamate concentrations in the NAcc[4,5], a brain region associated with the reinforcing and locomotor effects of cocaine[6,7]. Systemic and brain injections of non-selective mGluR agonists and antagonists mediate baseline and psychostimulant-induced locomotor activity[1–3]. mGluR5 is highly expressed in the NAcc[8], and repeated systemic cocaine treatment increases mGluR5 mRNA levels in the NAcc shell and dorsolateral striatum[9]; however, the functional role of mGluR5 in cocaine effects remains unknown.

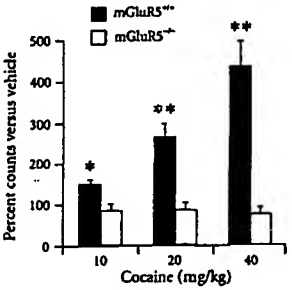

Fig. 1. Locomotor response to cocaine in mGluR5 WT (n = 14) and null mutant (n = 16) mice. Values represent mean percent activity counts ± s.e.m. *p < 0.05 versus saline; **p < 0.01 versus saline (Dunnett's test after two-way repeated-measures analysis of variance; ANOVA). For detailed methods, see the supplementary information page of *Nature Neuroscience* online.

To investigate this role, we first examined the locomotor responses to cocaine in F5 generation mGluR5 WT and null mutant mice. Baseline locomotor activity did not differ between mutant and WT mice (mutant, 2710 ± 642; WT, 2393 ± 479, mean horizontal activity counts/45-min session ± s.e.m.). Cocaine induced a significant, dose-dependent increase in locomotor activity in WT mice, but did not alter locomotor activity in mutant mice at any time point or dose tested (Fig. 1). Although mice received repeated cocaine exposure that may have induced some behavioral sensitization in WT mice, locomotor activity was not increased in mutant mice. Our results indicate that mGluR5 is essential for cocaine-induced hyperactivity.

To investigate whether the reinforcing properties of cocaine were affected by the mGluR5 mutation, we examined intravenous cocaine self-administration (SA) in WT and mutant mice. Acquisition of a discriminated two-lever food-reinforced task did not differ between WT and mutant mice (Fig. 2a). When intravenous cocaine was substituted for food, WT mice acquired stable cocaine SA across a typical dose range[10], but mutant mice did not self-administer cocaine at any dose tested (Fig. 2b). Active lever responding in mutant mice extinguished within three to five sessions at all cocaine doses, and no mutant mouse acquired stable SA, suggesting that the reinforcing properties of cocaine are absent in mice lacking mGluR5. Data from the food training suggest that the failure to acquire cocaine SA was not due to an inability to learn the lever-press task, and that the reinforcing properties of food are unchanged in mutant mice.

The selective mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP)[11] dose-dependently decreased cocaine SA (Fig. 2c) in C57Bl/6J mice. The effect of MPEP was specific to cocaine reinforcement, as MPEP had no effect on the rate of food-reinforced lever pressing (Fig. 2d) under the same schedule of reinforcement as during cocaine SA. These results suggest that

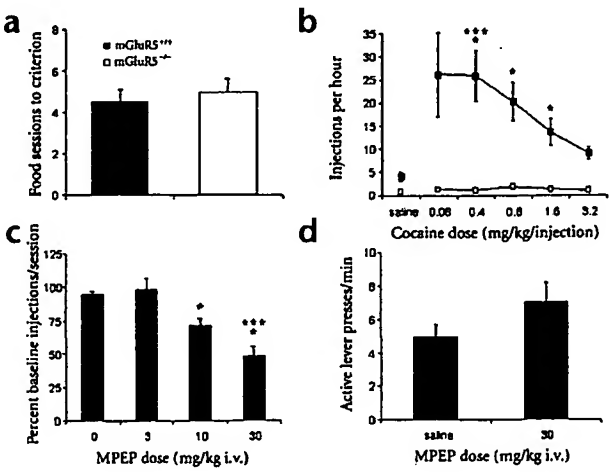

Fig. 2. Food and cocaine reinforcement. (**a**) Acquisition to criterion of food-reinforced lever pressing did not differ in mGluR5 WT (n = 5) and null mutant (n = 6) mice. Values represent mean ± s.e.m. (Student's t-test). (**b**) Cocaine SA in mice shown in (**a**). Values represent mean number of injections for 2 sessions at each dose ± s.e.m. *p < 0.05 versus saline within genotype; ***p < 0.05 WT at 0.4 versus WT at 3.2 mg/kg/injection (Bonferroni-corrected Student's t-tests after two-way repeated-measures ANOVA). (**c**) MPEP dose-dependently decreased cocaine SA in C57Bl/6J mice (n = 5). Values represent mean percent of baseline number of injections per 1 h session at 0.8 mg/kg/injection, *p < 0.05 versus saline; ***p < 0.05 versus 3 mg/kg MPEP (means comparisons after one-way ANOVA). (**d**) MPEP (30 mg/kg i.v.) had no effect on the number of food-reinforced lever presses per minute in C57Bl/6J mice (n = 5) (repeated-measures t-test). For detailed methods, see the supplementary information page of *Nature Neuroscience* online.

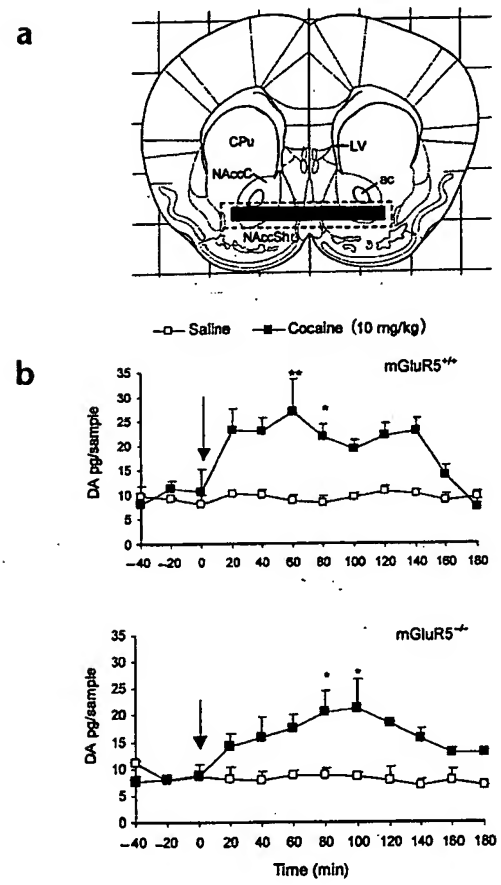

Fig. 3. Extracellular NAcc DA levels in mGluR5 WT and null mutant mice. (a) Dialysis probe location. Solid and dashed boxes indicate the minimum and maximum extent of probe placements. ac, anterior commissure; CPu, caudate putamen; LV, lateral ventricle; NAccC, nucleus accumbens core; NAccSh, nucleus accumbens shell. (b) DA level analysis. 10 µl samples were collected every 20 min from WT and mutant mice (saline, n = 3/genotype; cocaine, n = 3/genotype) and were analyzed by HPLC. Values represent mean pg/sample DA ± s.e.m. *$p < 0.05$; **$p < 0.01$ cocaine versus saline at the same time point within genotype (Bonferroni-corrected Student's t-test after separate two-way repeated-measures ANOVA). Extracellular DA levels were not significantly different between cocaine-treated WT and mutant groups (repeated-measures ANOVA, no significant main effects or interaction). For detailed methods, see the supplementary information page of *Nature Neuroscience* online.

the absence of cocaine SA in mutant mice was due to the loss of mGlu5 receptors and not to developmental alterations resulting from the genetic mutation.

Numerous studies have reported that self-administered cocaine increases DA levels in the ventral striatum[6], and psychostimulant-induced locomotor activity and increased mesoaccumbens DA levels in mice are closely correlated[12]. Because mutant mice showed neither a cocaine-induced locomotor response nor cocaine SA, we examined the effect of cocaine on NAcc DA levels using microdialysis in conscious, freely moving WT and mutant mice[12]. Wild-type and mutant mice had similar basal levels of extracellular DA. Cocaine-induced (10 mg/kg, i.p.) increases in extracellular DA levels did not differ between WT and mutant mice (Fig. 3b). These results suggest that the absence of mGluR5 affects neither baseline nor cocaine-induced increases in NAcc DA levels.

To ensure that changes in responses to cocaine in mutant mice were not due to mGluR5 mutation-induced alterations in dopaminergic elements, we investigated the brain distribution and expression of DA receptors and the DA transporter (DAT) in mutant and WT mice. No differences were found between WT and mutant mice in binding of the selective radiolabeled compounds ^{3}H-SCH23390 to D1-like and ^{3}H-YMO91512 to D2-like DA receptors and 3-WIN35,428 to the DAT, or in expression of D1 or D2 DA receptor mRNA examined by *in situ* hybridization (data not shown). These findings indicated that the expression and distribution of DA receptors and of the DAT are not altered in the mutant mice.

Several neurotransmitters and peptides contribute to cocaine dependence[6,7]. Although evidence supports a primary involvement for DA, the precise roles of specific DA receptor subtypes remain unclear. D1- or D2-like DA receptor antagonists reduce the reinforcing effects of cocaine[6,7]; however, D1 receptor mutant mice acquire a cocaine-conditioned place preference[13] and D2 receptor mutant mice self-administer cocaine (S.B. Caine *et al.*, *Soc. Neurosci. Abstr.* 26, 681.8, 2000). Likewise, the exact mechanisms of the mGluR5 contribution to cocaine dependence are not known. It is possible that glutamate acts in synergy with mesolimbic DA afferents into the NAcc to mediate the effects of cocaine. Acute cocaine increases extracellular NAcc dopamine[14] and glutamate[4] levels, and these effects are enhanced after repeated cocaine administration[5,14]. Excitatory amino acid (most likely glutamatergic) and mesolimbic dopaminergic terminals form synapses on single NAcc neurons[2,3]. Nucleus accumbens output neurons express both DA[2,6,7] and mGlu5[8] receptors. As has been suggested for locomotor activity[2], mGluR subtypes expressed by NAcc projection neurons may interact with dopaminergic inputs through their respective intracellular signaling pathways to influence the reinforcing effects of cocaine. Regardless of the specific mechanisms involved, the present results suggest that mGluR5 is essential in cocaine SA and locomotor effects.

Note: Supplementary methods are available on the Nature Neuroscience web site (http://neuroscience.nature.com/web_specials).

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Attenuation of morphine withdrawal symptoms by subtype-selective metabotropic glutamate receptor antagonists

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1 We have previously shown that chronic antagonism of group I metabotropic glutamate receptors (mGluRs), in the brain, attenuates the precipitated morphine withdrawal syndrome in rats. In the present investigation we assessed the effects of chronic antagonism of group II and III mGluRs on the severity of withdrawal symptoms in rats treated chronically with subcutaneous (s.c.) morphine.

2 Concurrently with s.c. morphine we infused intracerebroventricularly (i.c.v.) one of a series of phenylglycine derivatives selective for specific mGluR subtypes. Group II mGluRs ($mGluR_{2,3}$), which are negatively coupled to adenosine 3': 5'-cyclic monophosphate (cyclic AMP) production, were selectively antagonized with 2s, 1's, 2's-2-methyl-2-(2'-carboxycyclopropyl) glycine (MCCG). Group III mGluRs ($mGluR_{4,6,7\ and\ 8}$), which are also negatively linked to cyclic AMP production, were selectively antagonized with α-methyl-L-amino-4-phosphonobutanoate (MAP4). The effects of MCCG and MAP4 were compared with α-methyl-4-carboxyphenylglycine (MCPG), which non-selectively antagonizes group II mGluRs, as well as group I mGluRs ($mGluR_{1,5}$) which are positively coupled to phosphatidylinositol (PI) hydrolysis.

3 Chronic i.c.v. administration of both MCCG and MAP4 significantly decreased the time spent in withdrawal, MCPG and MCCG reduced the frequency of jumps and wet dog shakes and attenuated the severity of agitation.

4 Acute i.c.v. injection of mGluR antagonists just before the precipitation of withdrawal failed to decrease the severity of abstinence symptoms. Rather, acute i.c.v. injection of MCCG significantly increased the time spent in withdrawal.

5 Our results suggest that the development of opioid dependence is affected by mGluR-mediated PI hydrolysis and mGluR-regulated cyclic AMP production.

Keywords: Opioid; morphine; metabotropic glutamate receptor; dependence; α-methyl-4-carboxyphenylglycine (MCPG); 2s, 1's, 2's-2-methyl-2-(2'-carboxycyclopropyl)-glycine (MCCG); α-methyl-L-amino-4-phosphonobutanoate (MAP4)

Introduction

Although opioid analgesics are widely used in the management of pain, repeated use may lead to the development of tolerance and dependence. Tolerance is indicated by a decreased efficacy of the drug after chronic use, thereby leading to the requirement for a higher dose to achieve the desired analgesic effect. Dependence is a continued need for the drug to maintain a state of physiological equilibrium, following repeated administration, and is evidenced by withdrawal symptoms when drug administration is terminated. Recent evidence supports the involvement of excitatory amino acid (EAA), or glutamate, receptors in the development of tolerance and dependence (Marek *et al.*, 1991a,b; Trujillo & Akil, 1991; Fundytus & Coderre, 1994). Specifically, we have previously shown that chronic i.c.v. administration of antagonists selective for metabotropic glutamate receptors (mGluRs) significantly attenuates the development of dependence to systemically administered morphine (Fundytus & Coderre, 1994).

Metabotropic glutamate receptors are directly linked to intracellular second messenger systems via guanine nucleotide regulatory proteins (G proteins; Sladeczek *et al.*, 1985; Sugiyama *et al.*, 1987). Several subtypes of mGluRs, from $mGluR_1$ to $mGluR_8$, have been cloned (Nakanishi, 1992; Schoepp & Conn, 1993; Okamoto *et al.*, 1994; Saugstad *et al.*, 1994; Duvoisin *et al.*, 1995). The subtypes of mGluRs can be divided into groups based on receptor pharmacology, signalling cascades and sequence similarities (Hayashi *et al.*, 1994). The first group of mGluRs consists of $mGluR_1$ and $mGluR_5$, which are positively linked to the phosphatidylinositol second messenger system (Schoepp & Conn, 1993; Hayashi *et al.*, 1994). Activation of these receptors leads to phospholipase C (PLC)-mediated phosphatidylinositol (PI) hydrolysis. The second group of mGluRs consists of $mGluR_2$ and $mGluR_3$, which are negatively coupled, via adenylate cyclase, to the production of adenosine-3':5'-cyclic monophosphate (cyclic AMP) (Hayashi *et al.*, 1994). The third group of mGluRs consist of $mGluR_{4,6,7\ and\ 8}$, which are also negatively coupled to cyclic AMP production, but which show a different receptor pharmacology than $mGluR_{2,3}$ in that they are selectively activated by L-amino-4-phosphonobutanoate (L-AP4) (Hayashi *et al.*, 1994; Saugstad *et al.*, 1994).

In a previous study (Fundytus & Coderre, 1994), we showed that chronic intracerebroventricular (i.c.v.) infusion of the mGluR antagonist (*S*)-4-carboxyphenylglycine ((*S*)-4C-PG) concurrently with systemic morphine attenuated the development of morphine dependence. Although (*S*)-4C-PG selectively antagonizes group I mGluRs ($mGluR_1$ and $mGluR_5$), it has a secondary effect whereby it activates group II mGluRs ($mGluR_2$, $mGluR_3$) (Eaton *et al.*, 1993; Hayashi *et al.*, 1994; Watkins & Collingridge, 1994). It is therefore not entirely clear whether antagonism of $mGluR_{1,5}$ or activation of $mGluR_{2,3}$ is primarily responsible for the ability of (*S*)-4C-PG to attenuate the development of morphine dependence. To examine the relative contribution of each of the mGluR subtypes, we chose a range of phenylglycine antagonists selective to specific mGluRs. We examined how non-selective antagonism of group I ($mGluR_1$ and $mGluR_5$) and group II ($mGluR_2$ and $mGluR_3$) subtypes would affect the development of morphine dependence by administering α-methyl-4-carbox-

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1994; Thomsen *et al.*, 1994) i.c.v. concurrently with systemic morphine. We also selectively antagonized $mGluR_2$ and $mGluR_3$ with 2s,1's,2'S-2-methyl-2-(2'carboxycyclopropyl)-glycine(MCCG) (Jane *et al.*, 1994) and $mGluR_{4,\ 6-8}$ receptors with α-methyl-L-amino-4-phosphonobutanoate (MAP4) (Jane *et al.*, 1994), respectively. Furthermore, the effects of chronic treatment with these mGluR antagonists on morphine dependence were compared with the effects of acute treatment with the same agents given 10 min before naloxone. In the present study, we showed that chronic non-selective antagonism of mGluRs with MCPG, and chronic selective antagonism of either group II or III mGluRs significantly attenuates the development of morphine dependence.

Methods

Subjects and surgery

Subjects were male Long Evans hooded rats (Charles River, Quebec) weighing 280–350 g at the time of surgery. Rats were housed in groups of 2 to 4, maintained on a 12:12 h light:dark cycle (lights on at 06 h 00 min), and given food and water *ad libitum*.

On Day 0 rats were anaethestized with sodium pentobarbitone (Somnotol, MTC Pharmaceuticals, 60 mg kg^{-1}), and a 23 gauge stainless steel cannula was implanted stereotaxically in the lateral vertical of each rat (AP = −1.3 mm and L = −1.8 mm from bregma, and V = −3.8 mm (for chronic infusion) or V = −3.0 mm (for acute i.c.v. injections) from the top of the skull; Paxinos & Watson, 1986). For rats given chronic i.c.v. treatment, the cannula was attached to a Model 2001 Alzet osmotic pump filled with one of the antagonists or vehicle (dilute NaOH/saline). While the rats were still under pentobarbitone anaesthesia, one unprimed (not yet pumping) Model 2ML1 Alzet osmotic pump containing 50 mg ml^{-1} morphine sulphate (gift from Sabex, Quebec) solution was implanted subcutaneously (s.c.) on the back of each rat. Infusion of morphine began approximately 2 to 4 h following pump implantation. On the following day, Day 1, rats were briefly anaesthetized with halothane and a second unprimed Model 2ML1 Alzet pump containing 70 mg ml^{-1} morphine sulphate solution was implanted s.c. on the back of each rat. Once both pumps were in place, morphine sulphate was continuously infused s.c. at a rate of 10 μl h^{-1} from each pump, for a total dose of 36.65 μmol day^{-1} (28.8 mg day^{-1}). This two day pump implantation procedure was used to reduce the risk of mortality due to the accumulation of lethal systemic morphine concentrations before any tolerance development. Concurrently with morphine, the mGluR antagonists, MCPG ($n=20$), MCCG ($n=18$), MAP4 ($n=17$) (Tocris Cookson, Bristol, U.K.) or vehicle ($n=18$) were continuously infused at a rate of 1 μl h^{-1} in a dose of either 1.6, 8 or 40 nmol day^{-1} intracerebroventricularly (i.c.v.) in rats treated chronically with mGluR antagonists. To assess the effects of chronic administration of the selective mGluR antagonists on general (non-withdrawal) behaviour in rats not dependent on morphine, some rats were given 7 days of i.c.v. vehicle or 40 nmol day^{-1} of either MCPG ($n=3$), MCCG ($n=4$) or MAP4 ($n=4$) without concurrent morphine treatment. The effects of chronic administration of selective mGluR antagonists were also compared with effects of acute administration of the antagonists given as a single i.c.v. injection 10 min before the induction of withdrawal. Acute i.c.v. injections of either MCPG ($n=5$), MCCG ($n=6$) or MAP4 ($n=6$) were given in a dose of 2 nmol 4 μl^{-1}, or 4 μl vehicle ($n=15$), to rats that received chronic systemic morphine treatment as described above. A dose of 2 nmol was chosen to approximate the level received over a 1 to 2 h period in rats treated chronically with 40 nmol day^{-1}. Doses as high as 99 nmol i.c.v. have been used in learning experiments with no obvious side effects (Riedel & Reymann, 1993). The effects of acute administration of mGluR antagonists on general (non-withdrawal) behaviour were assessed by observing the behaviour of non-dependent rats after a single i.c.v. injection of either MCPG ($n=4$), MCCG ($n=4$) or MAP4 ($n=4$).

Measurement of withdrawal and non-withdrawal behaviours

Precipitated abstinence symptoms were assessed on the seventh day of treatment after injection of the opioid antagonist naloxone. Naloxone hydrochloride (Research Biochemicals Inc., Natick, MA) was injected s.c. in a volume of 1 ml kg^{-1} for a dose of 1 mg kg^{-1}. In chronically treated rats, behaviour was observed for 10 min before and 40 min after naloxone injection, during which time withdrawal symptoms were assessed by measuring the amount of time spent teeth chattering and writhing, as well as by counting jumps and wet dog shakes. In rats given an acute i.c.v. injection of mGluR antagonists, withdrawal behaviours were assessed for 10 min before the i.c.v. injection, 10 min after i.c.v. injection but before naloxone, and for 40 min after the injection of naloxone. For both chronic and acute conditions, agitation was assessed by rating the severity of vocalization upon light brushing of the back of the neck at both 20 and 40 min after the injection of naloxone. Severity of vocalization was rated on a scale of 0 to 3 where 0 = absent and 3 = severe. Also, for both chronic and acute i.c.v. treatment conditions, the time spent in withdrawal and non-withdrawal behaviours (ambulating, rearing, grooming and resting) was also measured for comparison in non-dependent rats (not given morphine) and morphine-dependent rats (given chronic s.c. morphine).

Statistical analysis

Timed withdrawal behaviours (teeth chattering, writhing) were analysed by 1 way ANOVA comparing the effects of various doses of each drug with the vehicle control group. Since all drugs were dissolved in the same vehicle, a single control group was used for each experiment to minimize the number of animals exposed to the full-fledged opioid withdrawal syndrome. Testing of the vehicle-treated rats was spread across the testing days for the experimental animals. Significant effects were further analysed by use of *post-hoc* LSD *t* tests. Counted withdrawal behaviours (number of jumps and wet dog shakes) and severity of agitation were analysed by a Kruskal-Wallis ANOVA for non-parametric data, followed by Mann-Whitney U-tests on significant main effects.

The effect of chronic antagonism of subtypes of mGluRs on non-withdrawal behaviours (ambulating, rearing, grooming and resting) was assessed by comparing the first two time blocks (i.e. 10 min before naloxone injection and 10 min after naloxone injection) for rats in each treatment group. The effects of acute i.c.v. injection of mGluR antagonists was assessed by comparing non-withdrawal and withdrawal behaviours for the first three times blocks (i.e. 10 min before i.c.v. injection, 10 min after i.c.v. injection but before naloxone injection, and 10 min after naloxone injection) in non-dependent and morphine-dependent rats. In both cases, a 3-way mixed ANOVA with i.c.v. treatment and morphine treatment as independent variables and time block as a repeated measure was performed on the % of time spent in each behaviour. Significant effects were further analysed with *post-hoc* LSD *t* tests.

Results

Figure 1 illustrates the severity of abstinence symptoms during the 40 min withdrawal period in rats chronically infused with s.c. morphine and either vehicle, MCPG, MCCG or MAP4 i.c.v. This experiment was performed to determine if chronic blockade of mGluRs would attenuate the development of morphine dependence. Chronic s.c. administration of 36.65 μmol day^{-1} morphine sulphate resulted in an intense

and reliable withdrawal syndrome, evidenced by the occurrence of teeth chattering, writhing, jumping, wet dog shaking and vocalization on touch (agitation) in vehicle-treated rats.

Figure 1a shows the amount of time spent in withdrawal (teeth chattering and writhing combined) during the 40 min withdrawal period for morphine-dependent rats treated concurrently with either i.c.v. vehicle, or 1.6, 8 or 40 nmol day^{-1} of either MCPG, MCCG or MAP4. ANOVA indicated a significant effect of MCCG ($F_{(3,32)}=4.14$, $P<0.05$) and MAP4 ($F_{3,31)}=4.62$, $P<0.05$), but not MCPG ($F_{3,34)}=1.90$, $P>0.05$). Both MCCG and MAP4 significantly decreased the time spent in withdrawal, as compared to the vehicle control group, at a dose of 1.6 nmol day^{-1}. Only MCCG was effective at 8 nmol day^{-1}, and only MAP4 was effective at 40 nmol day^{-1}. Although there was a trend for the non-selective mGluR antagonist MCPG to reduce the time spent in withdrawal, this effect failed to reach statistical significance.

Figure 1b illustrates the average frequency of counted symptoms (jumps and wet dog shakes combined) during the 40 min withdrawal period for morphine-dependent rats. Kruskal-Wallis ANOVA for non-parametric data indicated a significant effect of MCPG ($H_{(3,38)}=11.74$, $P<0.05$) and MCCG ($H_{(3,36)}14.14$, $P<0.01$), but not MAP4 ($H_{(3,35)}=6.08$, $P>0.05$). As indicated, MCPG significantly decreased the frequency of counted symptoms compared to the vehicle-treated control group at all doses used. MCCG significantly decreased the occurrence of counted symptoms at the highest dose, 40 nmol day^{-1}. Although there was a trend for MAP4 to decrease the frequency of control symptoms as dose was increased, this effect failed to reach statistical significance.

Figure 1c shows the average severity of agitation, as indicated by vocalization upon being lightly touched on the back of the neck, for morphine-dependent rats tested at 20 and 40 min post-naloxone. Kruskal-Wallis ANOVA for non-parametric data indicated a significant effect of MCPG ($H_{(3,38)}=11.65$, $P<0.05$), but not MCCG ($H_{(3,36)}=7.64$, $P>0.05$) nor MAP4 ($H_{(3,35)}=3.36$, $P>0.05$). The non-selective antagonist MCPG significantly decreased the severity of agitation at all doses used. Although MCCG appeared to attenuate the severity of agitation at 40 nmol day^{-1}, Kruskal-Wallis ANOVA failed to reach statistical significance. MAP4 did not significantly affect agitation at any of the doses used.

To verify that chronic infusion of mGluR antagonists had limited effects on general behaviour, non-withdrawal and withdrawal behaviours were compared during the 10 min before naloxone injection and the 10 min after naloxone injection for non-dependent and morphine-dependent rats chronically infused i.c.v. with either vehicle or 40 nmol day^{-1} of MCPG, MCCG or MAP4. Statistics confirmed that rats were more active earlier in the test session, with more time spent ambulating, rearing and grooming and less time spent resting, regardless of i.c.v. treatment (LSD t test, $P<0.05$). Before the injection of naloxone non-dependent and morphine-dependent rats behaved very similarly. After the injection of naloxone, non-dependent rats spent more time in non-withdrawal, and less time in withdrawal, behaviours than morphine-dependent rats (LSD t test, $P<0.05$). There were only a few effects of i.c.v. treatment on general activity level. Regardless of morphine treatment, i.c.v. MCPG- and MAP4-treated rats ambulated more than i.c.v. vehicle-treated rats ($P<0.05$, LSD t test). Also, non-dependent i.c.v. MCCG- and MAP4-treated rats reared more than non-dependent i.c.v. vehicle-treated rats ($P<0.05$, LSD t test) (data not shown).

Figure 2 depicts withdrawal symptoms during the 40 min withdrawal period for morphine-dependent rats given an acute i.c.v. injection of either vehicle or 2 nmol of either MCPG, MCCG or MAP4 10 min before naloxone injection. This experiment was performed to determine if acute blockade of mGluRs would decrease the expression of abstinence symptoms once dependence had developed.

Figure 2a illustrates the time spent in withdrawal (teeth chattering and writhing combined) during the 40 min withdrawal period for dependent rats given an acute i.c.v. injection

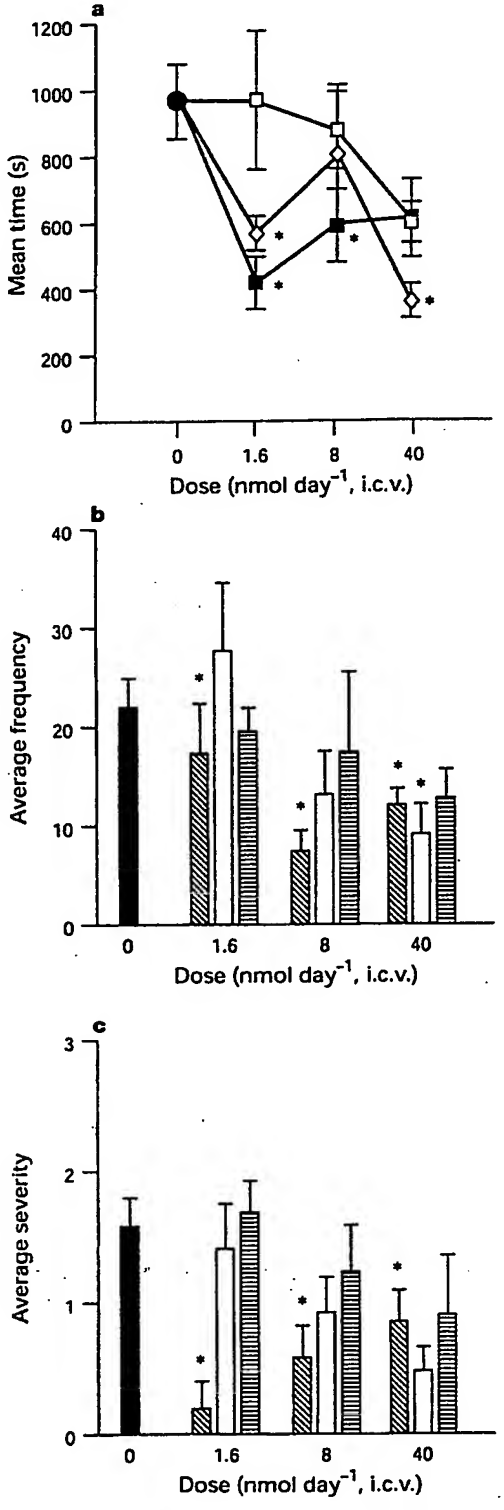

Figure 1 (a) Mean time spent in withdrawal (teeth chattering and writhing combined) during the 40 min withdrawal period for morphine-dependent rats treated chronically with either vehicle (●), MCPG (□), MCCG (■) or MAP4 (◇) i.c.v. Vertical lines show s.e.mean. *Significantly less than control ($P<0.05$, LSD t test). (b) Average frequency of counted symptoms (jumps and wet dog shakes combined) during the 40 min withdrawal period for morphine-dependent rats given either vehicle solid columns, MCPG diagonally-hatched columns, MCCG open columns or MAP4 horizontally-hatched columns. *Significantly less than control ($P<0.05$, Mann-Whitney U test). (c) Average severity of agitation during withdrawal for morphine-dependent rats given either vehicle solid columns, MCPG diagonally-hatched columns, MCCG open columns or MAP4 horizontally-hatched columns. *Significantly less than control ($P<0.05$; Mann-Whitney U test).

of either vehicle or 2 nmol of either MCPG, MCCG or MAP4. ANOVA indicated a significant effect of i.c.v. treatment ($F_{(3,28)}=4.20$, $P<0.01$). Acute injection of 2 nmol of MCCG 10 min before the precipitation of withdrawal significantly increased the time spent teeth chattering and writhing.

Figure 2b shows the average frequency of counted symptoms (jumps and wet dog shakes combined) during the 40 min withdrawal period for dependent rats given an acute i.c.v. injection of either vehicle, MCPG, MCCG or MAP4. Krusal-Wallis ANOVA for non-parametric data indicated that there were no differences between vehicle-treated rats and mGluR antagonist-treated rats ($H_{(3,32)}=0.40$ $P>0.05$).

Figure 2c shows the severity of agitation for dependent rats given an acute i.c.v. injection. Again, Krusal-Wallis ANOVA for non-parametric data indicated that there were no differences between vehicle-treated and mGluR antagonist-treated rats ($H_{(3,32)}=1.84$, $P>0.05$).

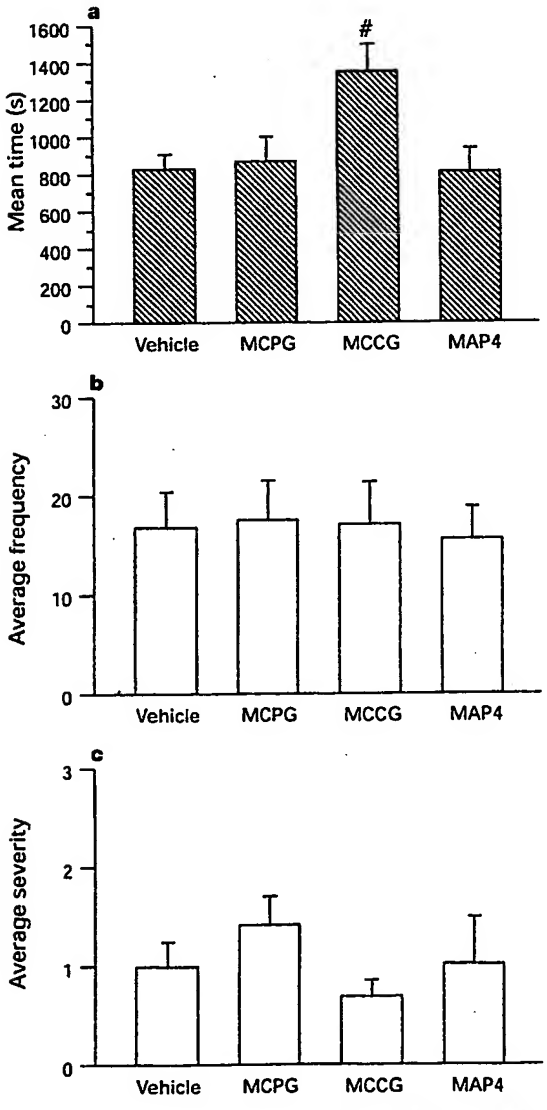

Figure 2 (a) Mean time spent in withdrawal (teeth chattering and writhing combined) during the 40 min withdrawal for morphine-dependent rats give an acute i.c.v. injection of either vehicle, or 2 nmol of MCPG, MCCG or MAP4 10 min before the precipitation of withdrawal. Vertical lines show s.e.mean #Significantly greater than vehicle (LSD t test, $P<0.05$) (b) Average frequency of counted symptoms (jumps and wet dog shakes combined) during the 40 min withdrawal period for morphine-dependent rats given an acute i.c.v. injection of either vehicle, MCPG, MCCG or MAP4 just before the precipitation of withdrawal. (c) Average severity of agitation for morphine-dependent rats given an acute i.c.v. injection of either vehicle, MCPG, MCCG or MAP4.

To verify that acute i.c.v. injection of mGluR antagonists had no significant effects on general behaviour, non-withdrawal and withdrawal behaviours were compared during the 10 min before i.c.v. injection, the 10 min after i.c.v. injection but before naloxone, and the 10 min after naloxone injection in non-dependent and morphine-dependent rats given an acute i.c.v. injection of either vehicle or 2 nmol of MCPG, MCCG or MAP4. Statistics confirmed that rats were more active in earlier time blocks (exhibiting more ambulating, rearing and grooming, and less resting) (LSD t test, $P<0.05$), and that after the injection of naloxone, morphine-dependent rats exhibited significantly more time in withdrawal and therefore less time in non-withdrawal behaviours than non-dependent rats (LSD t test, $P<0.05$). The only difference between vehicle-treated and mGluR antagonist-treated rats was that rats given MCCG exhibited significantly more time in withdrawal than vehicle-treated rats after the injection of naloxone ($P<0.05$, LSD t test) (data not shown).

Discussion

In the present study we have shown that chronic antagonism of mGluRs in general, as well as chronic antagonism of group II or III mGluRs, attenuates the severity of the precipitated morphine withdrawal syndrome. Non-selective antagonism of mGluRs with MCPG decreased the frequency of jumps and wet dog shakes, as well as the severity of agitation. Selective chronic antagonism of $mGluR_2$ and $mGluR_3$ with MCCG significantly decreased the time spent teeth chattering and writhing, the frequency of jumps and wet dog shakes, and the severity of agitation. Selective chronic antagonism of $mGluR_{4,6,7 \text{ and } 8}$ with MAP4 decreased the time spent teeth chattering and writhing. Although there was also a trend for MAP4 to decrease the frequency of jumps and wet dog shakes, this effect failed to reach significance. Acute i.c.v. administration of mGluR antagonists just before the precipitation of withdrawal failed to decrease the severity of abstinence symptoms, and in the case of MCCG, it actually increased the severity of the withdrawal syndrome.

Despite the fact that chronic administration of each antagonist reduced precipitated withdrawal symptoms, we did not observe a clear dose-response relationship for any of the agents. Perhaps this is due to the fact that our dose range was relatively small and the doses may have been in the same effective range. However, we avoided higher doses to prevent non-selective effects. MCPG has been shown to antagonize selectively group I and II mGluRs, with no effects on ionotropic glutamate receptors at concentrations up to 1 mM *in vitro* (Thomsen *et al.*, 1994). It has been shown that MCCG is selective for group II mGluRs and MAP4 is selective for group III mGluRs at concentrations up to 300 μM *in vitro* (Jane *et al.*, 1994), and had no effect on ionotropic receptors at concentrations as high as 500 μM (Jane *et al.*, 1994). Although our highest infusion (40 nmol day^{-1}) is a higher concentration (1667 μM), our two lower doses (1.6 and 8 nmol day^{-1}) are within the selective concentration range (66.67 and 333.3 μM, respectively). Therefore, because the effects were evident at these lower doses, they are probably due to actions at mGluRs. There were also differences in the degree to which each antagonist affected the various behaviours. This could possibly be due to inter-subject variability, or possibly because some minor or recessive withdrawal symptoms will sometimes increase as major or dominant symptoms decrease.

While chronic i.c.v. antagonism of mGluRs effectively decreased the severity of the withdrawal syndrome, there were few effects on non-withdrawal behaviours. The only effect due to i.c.v. treatment was that antagonist-treated rats were somewhat more active than vehicle-treated rats. However, the most significant changes in general behaviours were completely independent of treatment given. Thus, rats, including those treated only with vehicle, were generally less

active later in the test session because by this time they had explored the test box and were habituated to the environment.

The ability of chronic i.c.v. administration of MCCG and MAP4 to decrease the severity of morphine withdrawal suggests a role for mGluR-regulated cyclic AMP production in the development of opioid dependence. MCCG selectively antagonizes group II mGluRs ($mGluR_2$ and $mGluR_3$), and MAP4 selectively antagonizes group III mGluRs ($mGluR_{4,6,7 \text{ and } 8}$), both of which are negatively coupled to cyclic AMP production. There is a high expression of mRNA for group II and III mGluRs, as well as opioid receptors, in thalamus, striatum and cortex (Masu *et al.*, 1994; Mansour *et al.*, 1995). Because activity at opioid receptors also effects cyclic AMP production, it can be hypothesized that group II and III mGluRs interact with opioid receptors via actions on cyclic AMP production. It is generally accepted that chronic opioid treatment leads to compensatory changes in cyclic AMP in neuronal tissues. Thus, whereas acute administration of μ- and δ-opioids decreases cyclic AMP production, during chronic treatment cyclic AMP production returns to near control levels, and is greatly enhanced during withdrawal (Childers, 1991). We propose that by antagonizing group II or III mGluRs, we are removing one source by which the production of cyclic AMP is decreased, thereby modulating the chronic effects of opioids and possibly eliminating the need for the elicitation of compensatory mechanisms.

As well as antagonizing group II mGluRs, MCPG also antagonizes group I mGluRs ($mGluR_1$ and $mGluR_5$) which are positively coupled to PI hydrolysis. In a previous study (Fundytus & Coderre, 1994), we showed that selective antagonism of group I mGluRs with (*S*)-4C-PG attenuated the severity of morphine withdrawal. Because activity at opioid receptors also affects PI hydrolysis, these results suggest that mGluR-mediated changes in PI hydrolysis are also involved in the development of opioid dependence. There is a high level of expression of mRNA for opioid receptors, as well as group I mGluRs, in striatum and cortex (Masu *et al.*, 1994; Mansour *et al.*, 1995), suggesting that group I mGluRs and opioid receptors may interact. There is evidence that while acute administration of μ-opioids decreases PI hydrolysis, during chronic administration PI hydrolysis may increase to near control levels, and during withdrawal it is greatly enhanced (Dixon *et al.*, 1990; Barg *et al.*, 1994; Narita *et al.*, 1994; Busquets *et al.*, 1995), suggesting that compensatory mechanisms may be elicited during chronic opioid treatment. We propose that by chronically antagonizing group I mGluRs, we may be decreasing PI hydrolysis and thereby counteracting compensatory increases which may be elicited by chronic opioid treatment.

In summary, we have demonstrated the involvement of group II and III mGluRs, in addition to our previous evidence for a role of group I mGluRs, in the development of opioid dependence. Therefore, treatments which target mGluRs may be valuable tools in decreasing the incidence of opioid dependence.

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Presynaptic inhibitory action of the group II metabotropic glutamate receptor agonists, LY354740 and DCG-IV

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Abstract

Electrophysiological studies were carried out on the presynaptic inhibitory action of the group II metabotropic glutamate (mGlu) receptor agonists (+)-2-aminobicyclo[3.1.0]hexane-2-6-dicarboxylic acid (LY354740) and (2*S*,1′*R*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV) in three paths of the rat hippocampus, the medial and lateral perforant path to the dentate gyrus, and the Schaffer collateral/commissural path to CA1. LY354740 caused a dose-dependent reversible inhibition of the field excitatory postsynaptic potential (EPSP) in the medial and lateral perforant paths, with an EC_{50} of 115 ± 16 nM and 230 ± 58 nM, respectively. Maximal inhibition by LY354740 was much greater in the medial path (about 80%) than in the lateral path (about 50%). No inhibition was observed in CA1. A presynaptic inhibition was confirmed by LY354740 inducing dose-dependent changes in paired-pulse depression/facilitation. DCG-IV had a similar action to LY354740, but with a lower potency.

Keywords: Glutamate receptor, metabotropic; Perforant path; Paired-pulse

1. Introduction

The mediation of presynaptic inhibition by metabotropic glutamate (mGlu) receptors was first established in studies in which 1*S*,3*R*-aminocyclopentane-1,3-dicarboxylic acid (*trans*-ACPD) was shown to reversibly depress excitatory synaptic transmission in hippocampal CA1 (Baskys and Malenka, 1991; Desai and Conn, 1991; McGuinness et al., 1991; Pacelli and Kelso, 1991). A large number of studies have subsequently confirmed the presence of presynaptic mGlu receptors in many other areas of the brain, such as the hippocampal dentate gyrus (Macek et al., 1996; Bushell et al., 1996), neocortex (Sladeczek et al., 1993; Burke and Hablitz, 1994) and the striatum (Lovinger, 1991, Calabresi et al., 1992). Strong evidence that the inhibition of excitatory synaptic transmission was mediated presynaptically was shown by several lines of evidence. Firstly, inhibition by mGlu receptor agonists occurred without postsynaptic changes (Lovinger, 1991; Baskys and Malenka, 1991; Calabresi et al., 1992; Glaum et al., 1992; Lovinger et al., 1993; Burke and Hablitz, 1994); secondly, mGlu receptor activation reduced the AMPA- and NMDA-receptor mediated excitatory synaptic transmission with a similar potency (Baskys and Malenka, 1991; Lovinger, 1991; Pacelli and Kelso, 1991). Thirdly a change in paired-pulse facilitation or depression (indicative of a presynaptic modulation of transmitter release), was evoked by mGlu receptor agonists. Thus paired-pulse facilitation in CA1 was enhanced by mGlu receptor agonists (Baskys and Malenka, 1991; Burke and Hablitz, 1994; Gereau and Conn, 1995; Manzoni et al., 1997) and paired-pulse depression in the medial perforant path of the dentate gyrus being reduced by mGlu receptor agonists (Kahle and Cotman, 1993; Brown and Reymann, 1995).

Recent studies using a number of agonists selective for mGlu receptor group subtypes have shown the widespread presence of presynaptic group II mGlu receptors. In the hippocampus, a number of group II mGlu receptor selective agonists have been found to inhibit excitatory synaptic transmission, including (1*S*,3*S*)-1-aminocyclopentane-1,3-dicarboxylic acid {(1*S*,3*S*)-ACPD} (Vignes et al., 1995) and (2*S*,1′*R*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)-glycine (DCG-IV) (Yokoi et al., 1996) in young CA1; (2*S*,1′*S*,2′*S*)-2-carboxycyclopropyglycine (LCCG-1)

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(Ugolini and Bordi, 1995) and DCG-IV (Macek et al., 1996; Huang et al., 1997) in the medial perforant path of adult dentate gyrus, and DCG-IV (Macek et al., 1996; Bushell et al., 1996) and (1*S*,3*S*)-ACPD (Bushell et al., 1996) in the lateral perforant path of neonatal (Bushell et al., 1996) and adult (Macek et al., 1996) dentate gyrus.

(+)-2-Aminobicyclo[3.1.0]hexane-2-6-dicarboxylic acid (LY354740) is a recently synthesised high affinity efficacious and selective group II mGlu receptor agonist. (Bond et al., 1997; Monn et al., 1997; Schoepp et al., 1997a,b). LY354740 suppressed forskolin-stimulated cyclic 3′,5′-adenosine monophosphate (cAMP) formation at group II mGlu receptor with nanomolar potency, but had little or no agonist or antagonist action at group I mGlu receptor or group III mGlu receptor. The agent has potentially important clinical uses—it was found to prevent anxiety in the elevated plus maze and also prevent ACPD-induced limbic seizures. Moreover, it is orally active.

In the present study, we have investigated the presynaptic inhibitory action of LY354740 in the medial and lateral

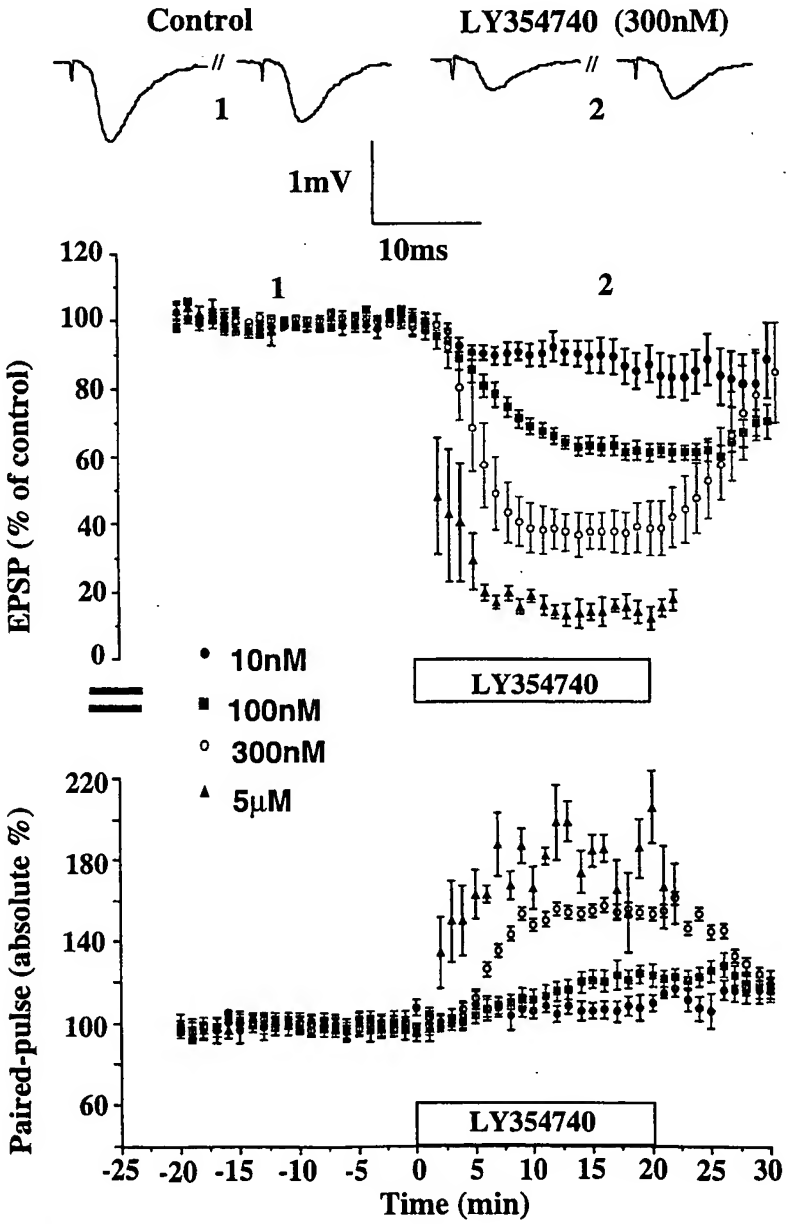

Fig. 1. LY354740 evokes a dose-dependent inhibition of excitatory synaptic transmission in the medial perforant path of the dentate gyrus in vitro. Upper graph, following a stable baseline for 20 min, perfusion of LY354740 at doses of 10 nM, 100 nM, 300 nM and 5 μM resulted in an increasing inhibition of the field EPSPs. Lower graph, 10 nM, 100 nM, 300 nM and 5 μM LY354740 caused an increasing reduction in paired-pulse depression accompanying the inhibition of the EPSP. The original traces show pairs of EPSPs in control and following application of 300 nM LY354740.

perforant path of the hippocampal dentate gyrus, and also CA1 hippocampus, comparing its action with the well established group II mGlu receptor agonist, DCG-IV.

2. Materials and methods

All experiments were carried out on hippocampal slices obtained from Wistar rats (50–70 g) (BioResources Unit, Trinity College, Dublin, Ireland). Slices were obtained as described previously (Huang et al., 1997). Briefly, the brain was rapidly removed after decapitation and placed in cold (5°C) oxygenated (95% O_2, 5% CO_2) artificial cerebro-spinal fluid (ACSF) containing in mM: NaCl, 120; $NaHCO_3$, 26; NaH_2PO_4, 1.25; KCl, 2.5; Mg_2SO_4, 2; $CaCl_2$, 2; glucose, 10). Hippocampal slices (350 μM) were cut using a Campden vibroslice (Campden Group Instruments, London, UK) and transferred immediately to an incubation chamber, maintained at room temperature, for a period of at least 60 min. Single slices were then transferred to a submersion type recording chamber at 30–31°C.

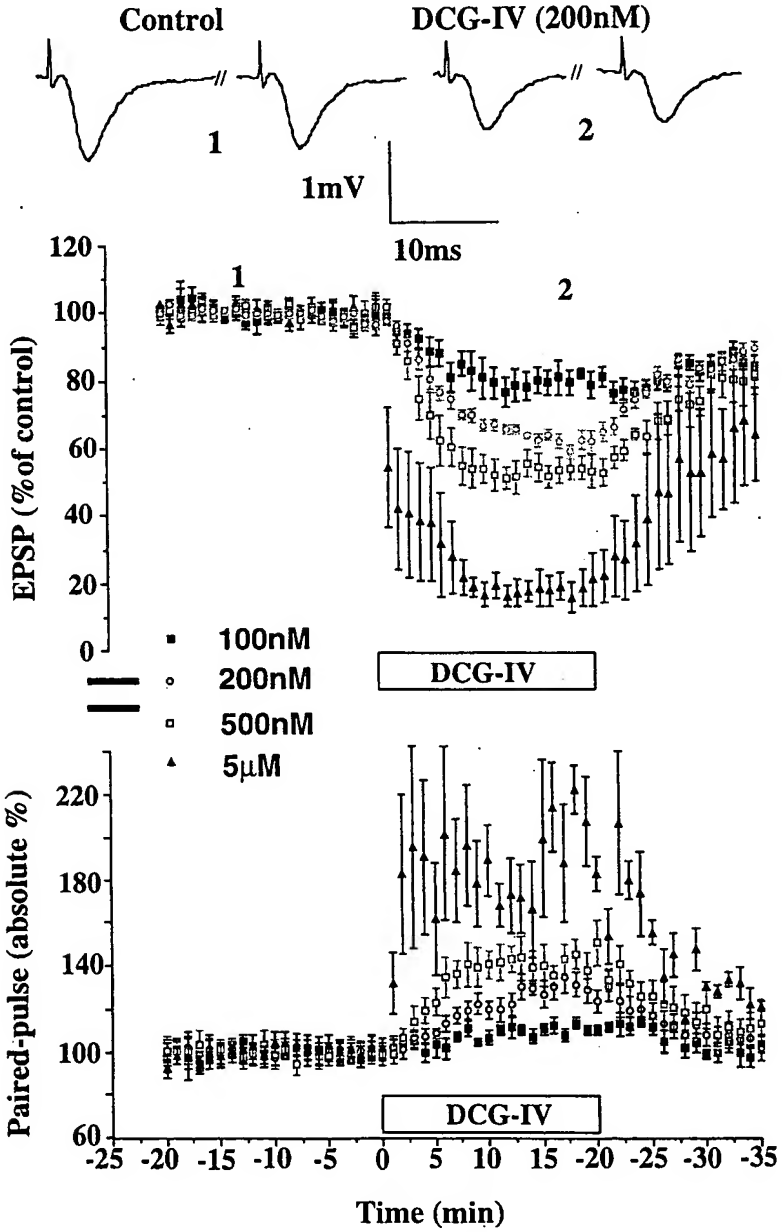

Fig. 2. DCG-IV evokes a dose-dependent inhibition of excitatory synaptic transmission in the medial perforant path of the dentate gyrus in vitro. Upper graph, following a stable baseline for 20 min, perfusion of DCG-IV at doses of 100 nM, 200 nM, 500 nM and 5 μM resulted in an increasing inhibition of the field EPSPs. Lower graph, 100 nM, 200 nM, 500 nM and 5 μM DCG-IV caused an increasing reduction in paired-pulse depression accompanying the inhibition of the EPSP. The original traces show pairs of EPSPs in control and following application of 200 nM DCG-IV.

Field excitatory postsynaptic potentials (EPSP) were recorded using standard glass electrodes filled with ACSF. Both recording and stimulating electrodes were placed in either the middle or outer third of the molecular layer of the dentate gyrus in order to stimulate and record from either the medial or lateral perforant path, respectively, and in the Schaffer collateral/commissural path in the stratum radiatum of CA1. Test EPSPs were evoked using a Grass S48 stimulator (0.0166 Hz, pulse width 0.1 ms) via a bipolar insulated tungsten wire electrode, adjusted to give about 30% of the maximal response (~1 mV). EPSP amplitude was measured using Maclab Scope, version 3.4. Paired-pulse stimulation (interstimulus interval of 40 ms) was applied in all experiments. For each pair, the amplitude of the second EPSP was divided by the first and multiplied by 100 to give the paired-pulse 'percentage'. A paired-pulse percentage of less than 100 was indicative of paired-pulse depression; a percentage greater than 100 was indicative of paired-pulse facilitation. The effect of LY354740 in the lateral perforant path and CA1 are presented in this way, in the text and in Figs. 3 and 5. However, the effects of LY354740 in the medial perforant path and also DCG-IV in the medial and lateral perforant path are represented in the text and Figs. 1, 2 and 4 as the 'absolute' percentage change from the normalised baseline control period, in order that the dose-dependent change in

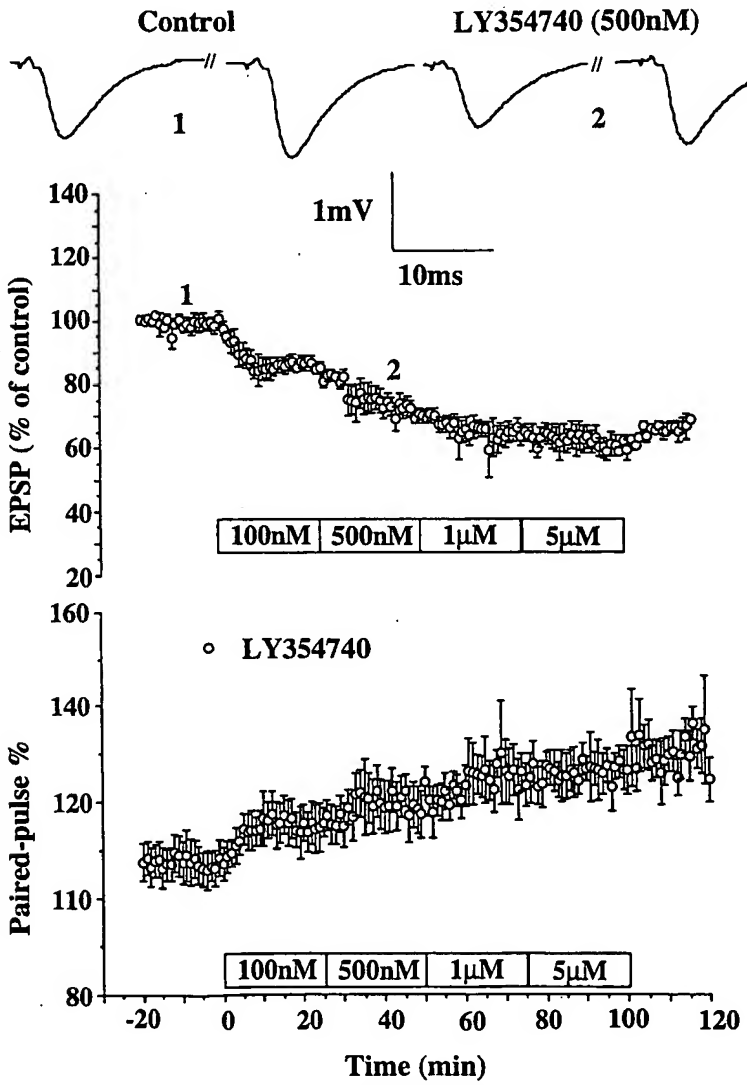

Fig. 3. LY354740 causes a dose-dependent inhibition of excitatory synaptic transmission in the lateral perforant path of the dentate gyrus in vitro. Upper graph, subsequent to a 20 min baseline period, perfusion of LY354740 at doses of 100 nM, 500 nM, 1 and 5 μM caused an increasing inhibition in the amplitude of the field EPSPs. Lower graph, paired-pulse facilitation in the lateral perforant path undergoes a concomitant increase in response to increasing doses of LY354740. The original traces show pairs of EPSPs elicited with a 40 ms inter-stimulus interval. Note the paired-pulse facilitation under control conditions, indicative of the lateral perforant pathway, and the subsequent increase in paired-pulse facilitation induced by LY354740 (500 nM).

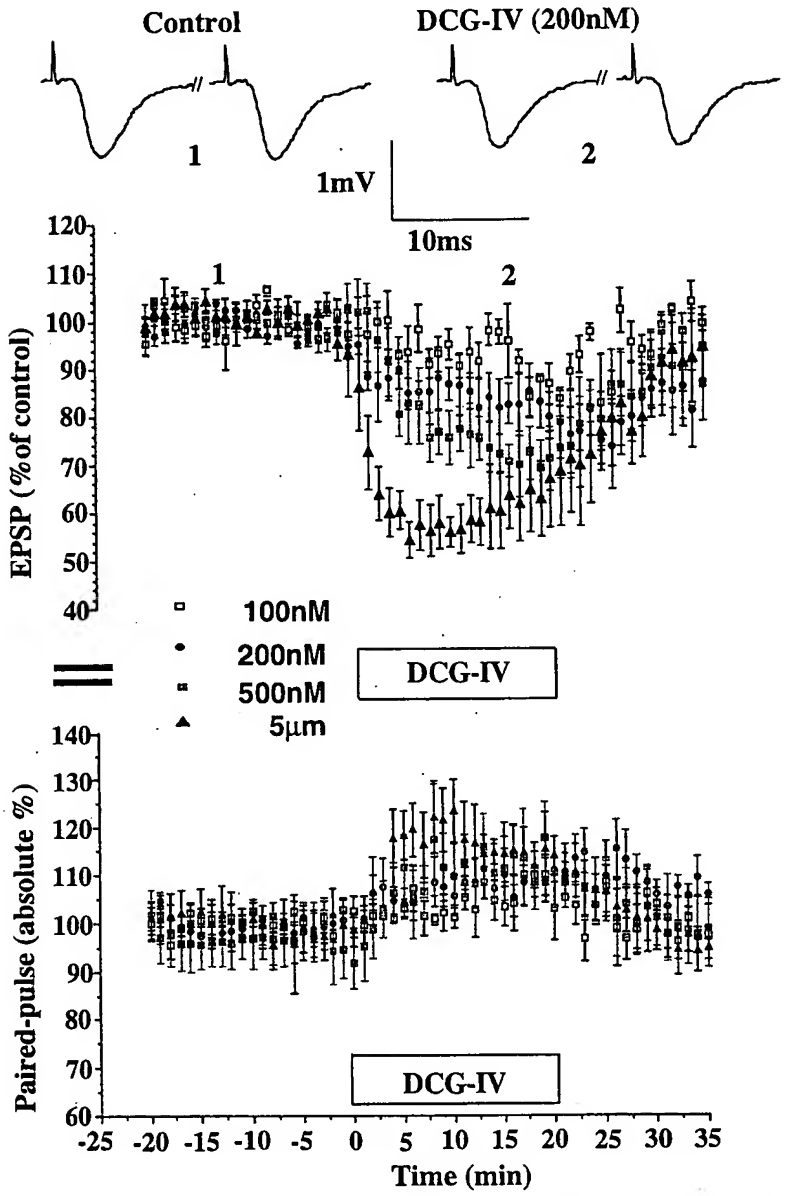

Fig. 4. DCG-IV causes a dose-dependent inhibition of excitatory synaptic transmission in the lateral perforant pathway of the dentate gyrus in vitro. Upper graph, following a stable baseline for 20 min, perfusion of DCG-IV at doses of 100, 200 and 500 nM and 5 μM caused a dose-dependent reduction in the EPSP amplitude. Lower graph, The absolute percentage changes in the level of paired-pulse facilitation induced by the application of stated doses of DCG-IV. The traces show the increase in paired-pulse facilitation in response to the application of 200 nM.

paired-pulse could be easily visualised in the figures with four different doses displayed (compare with Figs. 3 and 5).

All experiments in the dentate gyrus were carried out in the presence of 100 μM picrotoxin to block $GABA_A$ receptor-mediated inhibition. Drugs were added directly to the perfusate after establishing a steady baseline. Each slice was exposed to only one concentration of either LY354740 or DCG-IV except in some of the experiments carried out on LY354740 in the lateral perforant pathway in which cumulative doses were tested. LY354740 was a generous gift from Eli Lilly, USA. DCG-IV was obtained from Tocris Cookson.

Summarised results are expressed as normalised EPSP mean amplitude ± S.E.M. Data was analysed using Student's paired *t*-test, and repeated measures analysis of variance. Dose–response curves were constructed using Graphpad (Prism) software.

3. Results

3.1. LY354740 and DCG-IV inhibit the EPSP in the medial perforant path

The placement of the recording and stimulating electrodes in the medial perforant path was verified by the

presence of paired-pulse depression of EPSPs in response to paired-pulse stimulation.

After establishing a stable amplitude test EPSP for at least 20 min, bath application of LY354740 (20–25 min) caused a dose-dependent and reversible inhibition of the EPSP amplitude in the medial perforant path. The approximate threshold concentration of LY354740 was 10 nM, which induced a small inhibition of the EPSP amplitude of $11 \pm 4\%$ ($P < 0.05$, $n = 5$). LY354740 at higher concentrations of 100 nM and 300 nM reduced the EPSP amplitude by $36\% \pm 2\%$ ($P < 0.05$, $n = 7$) and $62 \pm 6\%$ ($P < 0.05$, $n = 4$), respectively (Fig. 1). Maximal inhibition of $85 \pm 3\%$ ($P < 0.05$, $n = 4$) was evoked with 5 μM LY354740. The dose–response curve for LY354740 (Fig. 6) was best fitted with a one-site binding hyperbola. The EC_{50} was estimated to be 115 ± 16 nM from this dose–response curve.

Evidence that the LY354740-evoked inhibition of the EPSPs was presynaptic was shown by an accompanying reduction in paired-pulse depression. In control media, EPSPs evoked in pairs, at an interval of 40 ms, resulted in paired-pulse depression of about 16%. Paired-pulse depression was reduced by LY354740 in a dose-dependent manner. Thus 10 nM, 100 nM, 300 nM and 5 μM caused absolute percentage changes in paired-pulse depression of $8 \pm 4\%$ ($P < 0.05$, $n = 5$), $21 \pm 12\%$ ($P < 0.05$, $n = 4$), $56 \pm 9\%$ ($P < 0.05$, $n = 4$) and $78 \pm 11.7\%$ ($P < 0.05$, $n = 5$), respectively (Fig. 1).

Perfusion of DCG-IV also resulted in dose-dependent and reversible inhibition of the EPSP amplitude, although less potently than LY354740. In the presence of 100 nM, 200 nM, 500 nM and 5μM DCG-IV, the EPSP amplitude was reduced by $21 \pm 4\%$ ($P < 0.05$, $n = 6$), $36 \pm 2\%$ ($P < 0.05$, $n = 8$), $47 \pm 3\%$ ($P < 0.05$, $n = 7$) and $82 \pm 4\%$ ($P < 0.05$, $n = 4$) of control values, respectively (Fig. 2, also see Fig. 4). The EC_{50} value was estimated to be 317 ± 54 nM from the dose–response curve of Fig. 6 fitted with a one-site binding hyperbola. Similar to that observed with LY354740, the inhibition of the test EPSP by DCG-IV was accompanied by a reduction in paired-pulse depression. For example, in 100 nM, 200 nM, 500 nM and 5 μM, the absolute percentage changes in paired-pulse were

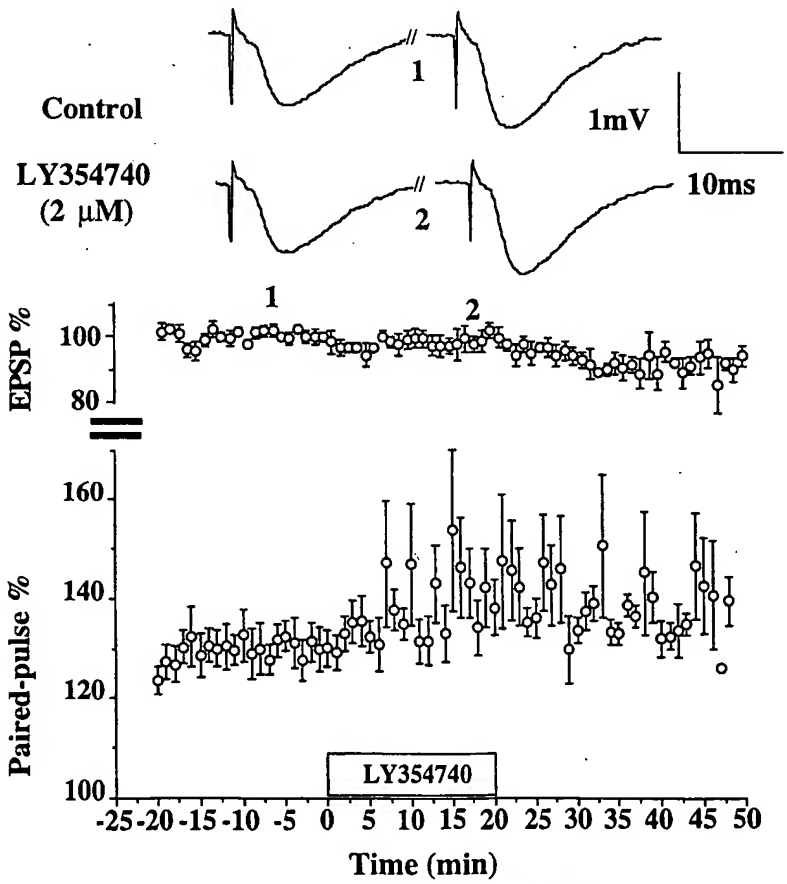

Fig. 5. Dose–response curves for the action of LY354740 and DCG-IV in the medial and lateral perforant paths. The dose–response curves were best fitted with a one-site binding hyperbola (Prism software) $Y = B_{max} \cdot X/(K_d + X)$ where B_{max} is the maximal binding, and K_d is the concentration of ligand required to reach half-maximal binding. The EC_{50} for LY354740 was estimated to be 115 ± 16 nM and 230 ± 58 nM in the medial and lateral perforant paths, respectively. The EC_{50} for DCG-IV was estimated to be 317 ± 54 nM and 334 ± 69 nM in the medial and lateral perforant paths, respectively. Each point plots the mean ± S.E.M. values for four to eight slices.

11 ± 3% ($P < 0.05$, $n = 6$), 29 ± 6% ($P < 0.05$, $n = 7$), 42 ± 8% ($P < 0.05$, $n = 7$) and 89 ± 19% ($P < 0.05$, $n = 4$; Fig. 2), respectively.

3.2. LY354740 and DCG-IV inhibit the EPSP in the lateral perforant path

The placement of the recording and stimulating electrodes in the lateral perforant path was verified by the presence of paired-pulse facilitation of EPSPs in response to paired-pulse stimulation.

Both LY354740 and DCG-IV inhibited the EPSP in the lateral perforant path, but with a lower potency and lower maximal inhibition than in the medial perforant path. The EPSP amplitude was significantly reduced by 15 ± 2%, 28 ± 3%, 46 ± 4% and 48 ± 4% in 100 nM, 500 nM, 1 μM and 5 μM LY354740, respectively (Fig. 3, also see Fig. 6). All values were significant ($P < 0.01$, $n = 5$) when tested using a repeated measures analysis of variance. The EC_{50} value was estimated to be 230 ± 58 nM from the dose–response curve (Fig. 6). A dose-dependent significant increase in paired-pulse facilitation accompanied the LY354740-induced depression of the EPSPs, in the lateral perforant path. In control conditions the paired-pulse facilitation was 7 ± 4%. This increased to 15 ± 4%, 20 ± 4%, 25 ± 3% and 25 ± 4% in 100 nM, 500 nM, 1 μM and 5 μM, respectively. All values were significant when tested using the repeated measures analysis of variance ($P < 0.01$, $n = 5$) (Fig. 3).

DCG-IV also inhibited the EPSP in the lateral perforant path, but like the medial perforant path, with a lower potency than LY354740. Perfusion of DCG-IV reduced the EPSP amplitude by 7 ± 3%, 16 ± 5%, 17 ± 5% and 39 ± 7% in 100 nM, 200 nM, 500 nM and 5 μM, respectively. All values were significant when tested using the Student's paired t-test ($P < 0.01$, $n = 5$) (Fig. 4). The estimated EC_{50} value was 334 ± 69 nM. Like that with LY354740, the increase in paired-pulse facilitation was relatively small and was dose-dependent. DCG-IV caused an absolute percentage increase in paired-pulse facilitation of 7 ± 4%, 9 ± 4%, 13 ± 7% and 14 ± 6% in 100 nM, 200 nM, 500 nM and 5 μM, respectively. All values were significant when tested using the Student's paired t-test ($P < 0.01$, $n = 5$) (Fig. 4).

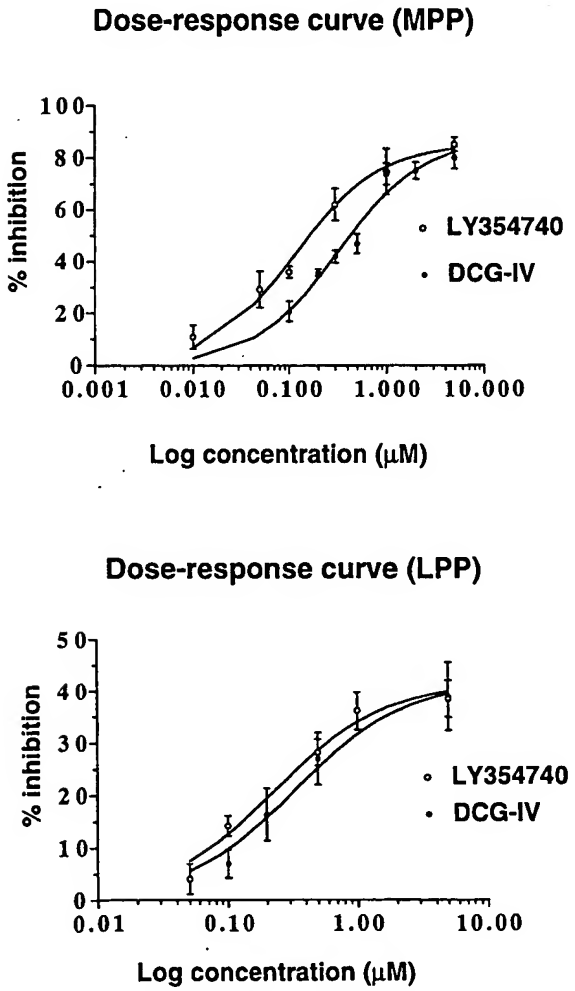

Fig. 6. LY354740 does not produce an inhibition of excitatory synaptic transmission in the CA1 hippocampus. Upper graph, application of 2 μM LY354740 does not produce an inhibition of the EPSP in CA1. Lower graph, LY354740 does not produce a significant change in paired-pulse facilitation in CA1.

3.3. LY354740 has no effect on EPSP amplitude in CA1

LY354740 was applied at a concentration which was sufficient to maximally depress synaptic transmission in both pathways of the dentate gyrus (2 μM). At this concentration LY354740 had no discernible effect, 98.9 ± 2.6% ($P > 0.05$, $n = 4$; Fig. 5).

4. Discussion

The present electrophysiological study has shown that LY354740 is a potent agonist at the group II mGlu receptor responsible for mediating inhibition of EPSPs at the medial perforant path in the dentate gyrus in vitro, with the threshold dose about 10 nM and the EC_{50} close to 100 nM. This is the most potent agonist action at group II mGlu receptors, demonstrated in electrophysiological studies, in this pathway. In comparison with other agonists, electrophysiological studies have shown that DCG-IV has an EC_{50} of close to 300 nM (present study), while LCCG-1 has an EC_{50} of 30 μM (Ugolini and Bordi, 1995). The particularly potent properties of LY354740 are in agreement with neurochemical studies in which LY354740 has been shown to be the most potent group II agonist synthesised, with an EC_{50} of 5 nM and 24 nM for the inhibition of forskolin-stimulated cAMP at expressed mGluR2 receptor and mGlu receptors, respectively (Schoepp et al., 1997b)

and a similar potency for group II mGlu receptor in the rat cerebral cortex and hippocampus group II mGlu receptor (Monn et al., 1997; Schoepp et al., 1997b).

Both LY354740 and DCG-IV were also found to cause a depression of the EPSP in the lateral perforant path, although this path was less sensitive to the agonists, the EC_{50} for LY354740 and DCG-IV being close to 200 nM and 300 nM, respectively, much higher than in the medial path. In addition, the maximal inhibition (40–50%) was much lower than in the medial path (80–90%). Such a reduced sensitivity in the lateral perforant path is likely to reflect a lower density of group II mGlu receptors in this path. A difference in the extent of inhibition by group II mGlu receptor agonists between the medial and lateral perforant path was shown previously by Macek et al. (1996), although this study also found a much lower sensitivity to DCG-IV in both the medial and lateral perforant path than the present study, with the EC_{50} for the action of DCG-IV being 1.6 μM and > 3 μM, respectively. This lower sensitivity in the study of Macek et al. (1996) may be due to a developmental decrease in group II mGlu receptor sensitivity, similar to that occurring in CA1 (Vignes et al., 1995; Shigemoto et al., 1997). Thus Macek et al. (1996) used animals of 100–150 g weight, compared to 50–70 g weight in the present study. However, we have not found any developmental decrease in sensitivity to group II mGlu receptor agonists in the medial perforant path of the dentate gyrus (unpublished results). The complete lack of effect of LY354740 in the CA1 region of the hippocampus reflects the absence of group II mGlu receptors in this region of the adult rat, and confirms previous electrophysiological studies using lower potency agonists than LY354740, such as DCG-IV (Gereau and Conn, 1995; Breakwell et al., 1997)

The inhibition of the EPSP by LY354740 and DCG-IV was accompanied by a change in paired-pulse depression/facilitation. Such changes demonstrate that the group II mGlu receptor agonists caused a decrease in the presynaptic probability of transmitter release. The most likely receptor mediating the presynaptic inhibition demonstrated in the present study is mGluR2. Several immunohistochemical studies have shown that mGluR2 is located at a very high density on perforant path axons in the molecular layer of the dentate gyrus, especially in the medial perforant path (Ohishi et al., 1993; Neki et al., 1996; Petralia et al., 1996; Shigemoto et al., 1997). We cannot rule out inhibition via mGluR3 receptors, although this receptor is only present at a much lower density in the hippocampus and is mainly located on glial cells (Petralia et al., 1996). Activation of the group II mGlu receptors may inhibit Ca^{2+} influx, thereby reducing the probability of transmitter release. Alternatively, as the mGluR2 receptors are located preterminally at some distance from the transmitter release sites and corresponding Ca^{2+} channels, rather than presynaptically (Shigemoto et al., 1997), activation of mGluR2 receptors may be linked to opening of K^+ channels, a reduction in the amplitude/duration of the axonal action potential, and thereby reduction of Ca^{2+} influx and the probability of transmitter release.

Acknowledgements

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Pharmacological Evidence for an Involvement of Group II and Group III mGluRs in the Presynaptic Regulation of Excitatory Synaptic Responses in the CA1 Region of Rat Hippocampal Slices

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Summary—The actions of four mGluR antagonists, (+)-MCPG, MAP4, MCCG and (*S*)-4CPG, were evaluated against agonist-induced depressions of synaptic transmission at the Schaffer collateral–commissural pathway in rat hippocampal slices. (+)-MCPG (1 mM) reversed very effectively depressions of field EPSPs induced by (1*S*,3*R*)-ACPD and (1*S*,3*S*)-ACPD but had weak and variable effects on depressions induced by L-AP4. It had no effect on depressions induced by either (−)-baclofen or carbachol. In contrast, MAP4 (500 μM) reversed very effectively depressions induced by L-AP4 without affecting depressions induced by (1*S*,3*S*)-ACPD. MCCG (1 mM) had the opposite activity; it antagonized depressions induced by (1*S*,3*S*)-ACPD but not those induced by L-AP4. Finally, (*S*)-4CPG (1 mM) reversed small depressions of field EPSPs induced by high concentrations (50–100 μM) of (1*S*,3*R*)- and (1*S*,3*S*)-ACPD, but not L-AP4, whilst having no effect on large depressions induced by 10 μM (1*S*,3*S*)-ACPD in voltage-clamped cells. These results confirm and extend the effectiveness and selectivity of (+)-MCPG as an mGluR antagonist. The divergent effects of the group I antagonist, (*S*)-4CPG, can be explained by an indirect action on postsynaptic receptors which is manifest when high agonist concentrations are used in non-voltage-clamp experiments. The action of MCCG and MAP4 indicates that two pharmacologically-distinct mGluRs, belonging to classes II and III, can regulate synaptic transmission in the CA1 region via presynaptic mechanisms.

Group I consists of mGluR5 and mGluR1

Keywords—Metabotropic glutamate receptors (mGluR), ACPD, L-AP4, (+)-MCPG, MCCG, (*S*)-4CPG, MAP4, hippocampus

Activation of metabotropic glutamate receptors (mGluRs) produces multiple effects within the CA1 region of the hippocampus, including a depression of excitatory synaptic transmission (Pin and Duvoisin, 1995). In particular, it has been shown that the mGluR agonists (1*SR*,3*RS*)-1-aminocyclopentane-1,3-dicarboxylate [(1*SR*,3*RS*)-ACPD], its active constituent (1*S*,3*R*)-ACPD (Irving *et al.*, 1990), and (*S*)-2-amino-4-phosphonobutanoate (L-AP4) depress synaptic transmission at Schaffer collateral–commissural synapses via a presynaptic site of action (Baskys and Malenka, 1991; Harvey *et al.*, 1991; Desai *et al.*, 1992). It has also been shown that the mGluR antagonist α-methyl-4-carboxyphenylglycine (MCPG) inhibits the presynaptic depressant actions of (1*S*,3*R*)-ACPD at this synapse (Davies *et al.*, 1993; Manzoni *et al.*, 1994; Bolshakov and Siegelbaum, 1994; Watkins and Collingridge, 1994).

In the present study, we have extended our analysis of the antagonist actions of (+)-MCPG (Davies *et al.*, 1993) on agonist-induced depressions of excitatory synaptic transmission, in the CA1 region of rat hippocampal slices. In addition, we have examined the effects of three antagonists which are selective/specific for the three mGluR subgroups; group I—(*S*)-4-carboxyphenylglycine [(*S*)-4CPG], group II—2*S*,1′*S*,2′*S*-2-methyl-2-(2′-carboxycyclopropyl)glycine (MCCG), and group III—(*S*)-2-methyl-2-amino-4-phosphonobutanoate (MAP4) (Jane *et al.*, 1994; Watkins and Collingridge, 1994).

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METHODS

Experiments were performed on hippocampal slices obtained from Wistar rats (approx 2 weeks old) as described previously (Davies *et al.*, 1990). Coronal slices (400 μm thick) containing hippocampus were cut using a Campden vibroslicer. The hippocampal region was dissected from these slices and area CA3 removed. The slices were stored at room temperature for at least 1 hr before being transferred to either an interface or submerged recording chamber where they were maintained at either 28°–32°C or at room temperature and perfused at a rate of approx 2 ml min^{-1} with medium which comprised (mM): NaCl 124; KCl 3; $NaHCO_3$ 26; $CaCl_2$ 2; $MgSO_4$ 1; D-glucose 10; NaH_2PO_4 1.25, bubbled with a 95% O_2/5% CO_2 mixture. Extracellular recordings of field excitatory postsynaptic potentials (fEPSPs) were obtained from *stratum radiatum* using glass microelectrodes (4 MΩ) filled with NaCl (4 M) connected to an Axoclamp-2 amplifier. Whole-cell patch-clamp recordings of excitatory postsynaptic currents (EPSCs) were obtained from *stratum pyramidale* using glass microelectrodes (5–7 MΩ; seal resistances approx 10 GΩ) filled with (mM): $CsMeSO_4$ 130; NaCl 1; $MgCl_2$ 1; $CaCl_2$ 0.035; EGTA 0.05; QX-314 5; HEPES 5 (pH 7.3) connected to an Axopatch-1D amplifier, as described previously (Clark and Collingridge, 1995). Neurones were voltage-clamped at −60 mV. EPSCs were isolated by the addition of picrotoxin (50 μM) to the perfusate and by the inclusion of Cs^+ in the patch pipette. Drugs were administered by addition to the superfusing medium and were applied for a sufficient period to allow their full equilibration.

RESULTS

Agonist actions

Field EPSPs, evoked by low frequency stimulation of the Schaffer collateral–commissural pathway, were depressed by (1*S*,3*R*)-ACPD (25–200 μM; $n = 37$) in a reversible manner. The mean ± SEM depressions were 31 ± 14% ($n = 18$) and 37 ± 12% ($n = 16$) for doses of 50 and 100 μM, respectively. In contrast, the presynaptic fibre volley was unaffected by (1*S*,3*R*)-ACPD. The threshold concentration was approx 25 μM [6 ± 3% depression ($n = 3$)] and the maximum effective concentration approx 100 μM. The depression induced by 100 μM was highly variable between preparations ranging between 9 and 90% but depressions ranging between 20 and 60% were selected to test the antagonists. In the continued presence of (1*S*,3*R*)-ACPD the depression was sustained. Similar sustained but reversible depressions were also induced by (1*S*,3*S*)-ACPD (10–150 μM; $n = 14$), depressions at 50 μM being 41 ± 6% ($n = 8$), and L-AP4 (25–200 μM; $n = 25$), depressions at 50 μM being 41 ± 4% ($n = 3$).

Actions of (+)-MCPG

(+)-MCPG (1 mM) had no effect on field EPSPs *per se* ($n = 3$) but invariably reversed the depressant effects of (1*S*,3*R*)-ACPD (50 μM; $n = 4$). In contrast, (−)-MCPG had no effect on depressions induced by (1*S*,3*R*)-ACPD (50 μM; $n = 2$). A comparison of the actions of the stereo-isomers of MCPG is illustrated in Fig. 1 and pooled data illustrating the

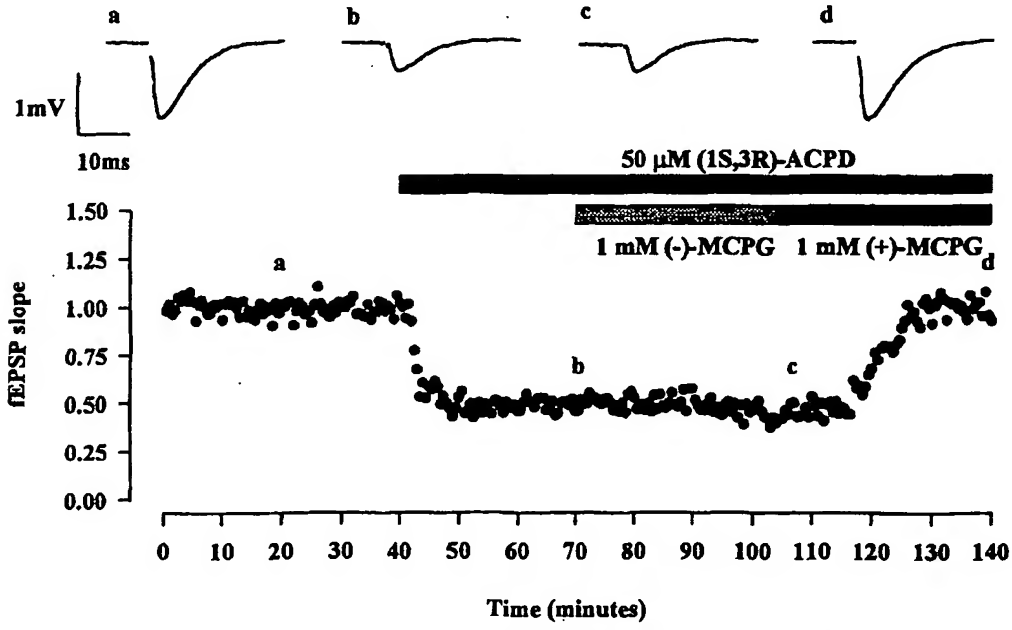

Fig. 1. Stereoselective reversal by MCPG of the depressant action of (1*S*,3*R*)-ACPD. The graph plots the slope of the field EPSP, normalized with respect to the entire illustrated baseline prior to drug administration, vs time. Drugs were applied for the times indicated by bars above the graph and representative traces are shown for the times indicated by a–d.

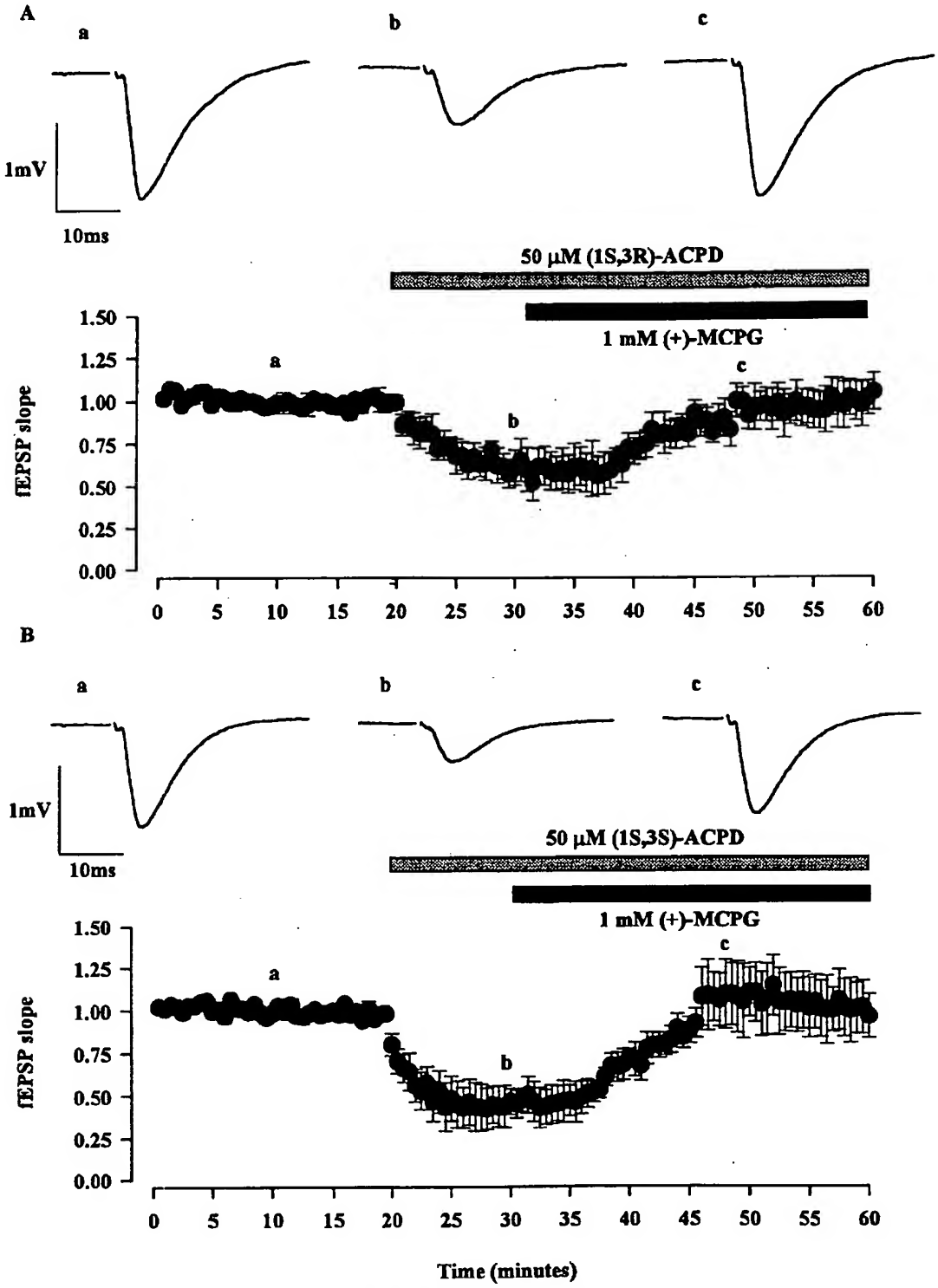

Fig. 2. Pooled data illustrating reversal of the depressant effects of (A) (1*S*,3*R*)-ACPD and (B) (1*S*,3*S*)-ACPD by (+)-MCPG. The graphs plot mean ± SEM for 4 and 3 slices, respectively, where the same protocol was adopted. (+)-MCPG (1 mM) was also tested against (1*S*,3*R*)-ACPD (50–100 μM) using a variety of different protocols and was effective in 7 of 8 slices tested.

effects of (+)-MCPG *versus* (1*S*,3*R*)-ACPD on all slices where the same protocol was tested is shown in Fig. 2(A). (+)-MCPG also reversed invariably the depressant effects of (1*S*,3*S*)-ACPD [50 μM; $n = 3$; Fig. 2(B)]. In contrast, (+)-MCPG had either a partial effect ($n = 3$) or no discernible action ($n = 3$) on depressions induced by L-AP4 (25 μM; Fig. 3). The actions of (+)-MCPG were selective towards mGluR-

mediated effects since (+)-MCPG had no effect on depressions induced by either carbachol [3–5 μM; $n = 3$; Fig. 4(A)] or (−)-baclofen [5 μM; $n = 3$; Fig. 4(B)].

Actions of MAP4

In contrast to (+)-MCPG, MAP4 (500 μM) reversed invariably the depressant actions of L-AP4 (25 μM; $n = 4$; Fig. 5). Since this is the first example of good antagonism of the actions of L-AP4 at this synapse we investigated the actions of MAP4 further, using whole-cell patch-clamp techniques. In these experiments possible indirect depressions of synaptic transmission caused by postsynaptic depolarizing actions of agonists were avoided (since the cells were dialysed with Cs^+ and voltage-clamped). In all neurones studied neither agonist tested affected holding current or input resistance. In agreement with the field potential data, MAP4 (500 μM) reversed the depression of EPSCs induced by L-AP4 (25 μM; $n = 6$; Fig. 6). In contrast, MAP4 had no effect on depressions induced by (1*S*,3*S*)-ACPD (25 μM; $n = 6$; Fig. 7). Furthermore, MAP4 was still inactive when tested at a higher concentration (1 mM) versus a lower concentration (10 μM) of (1*S*,3*S*)-ACPD ($n = 3$; data not shown).

Actions of MCCG

We next tested the effects of MCCG using whole-cell recording experiments. At a concentration of 1 mM, MCCG partially, but invariably, reversed the effects of (1*S*,3*S*)-ACPD [10 μM; $n = 8$; Fig. 8(A,C)] but had no effect on responses induced by L-AP4 [25 μM; $n = 3$; Fig. 8(B)]. The magnitude of the reversal by MCCG

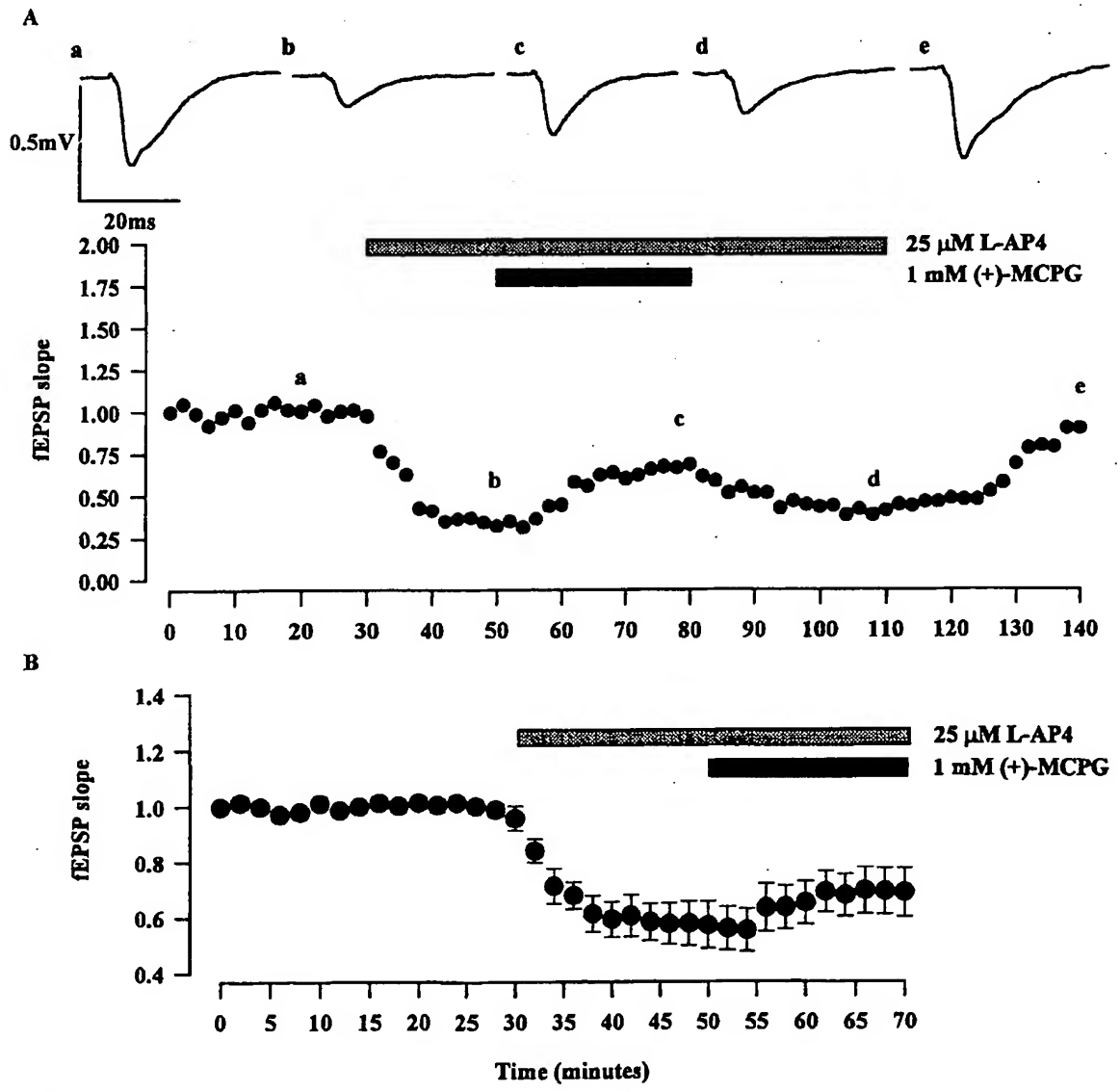

Fig. 3. Variable effects of (+)-MCPG on depressions induced by L-AP4. (A) shows an example of a small but reversible antagonism. (B) plots pooled data for all 6 slices using this protocol.

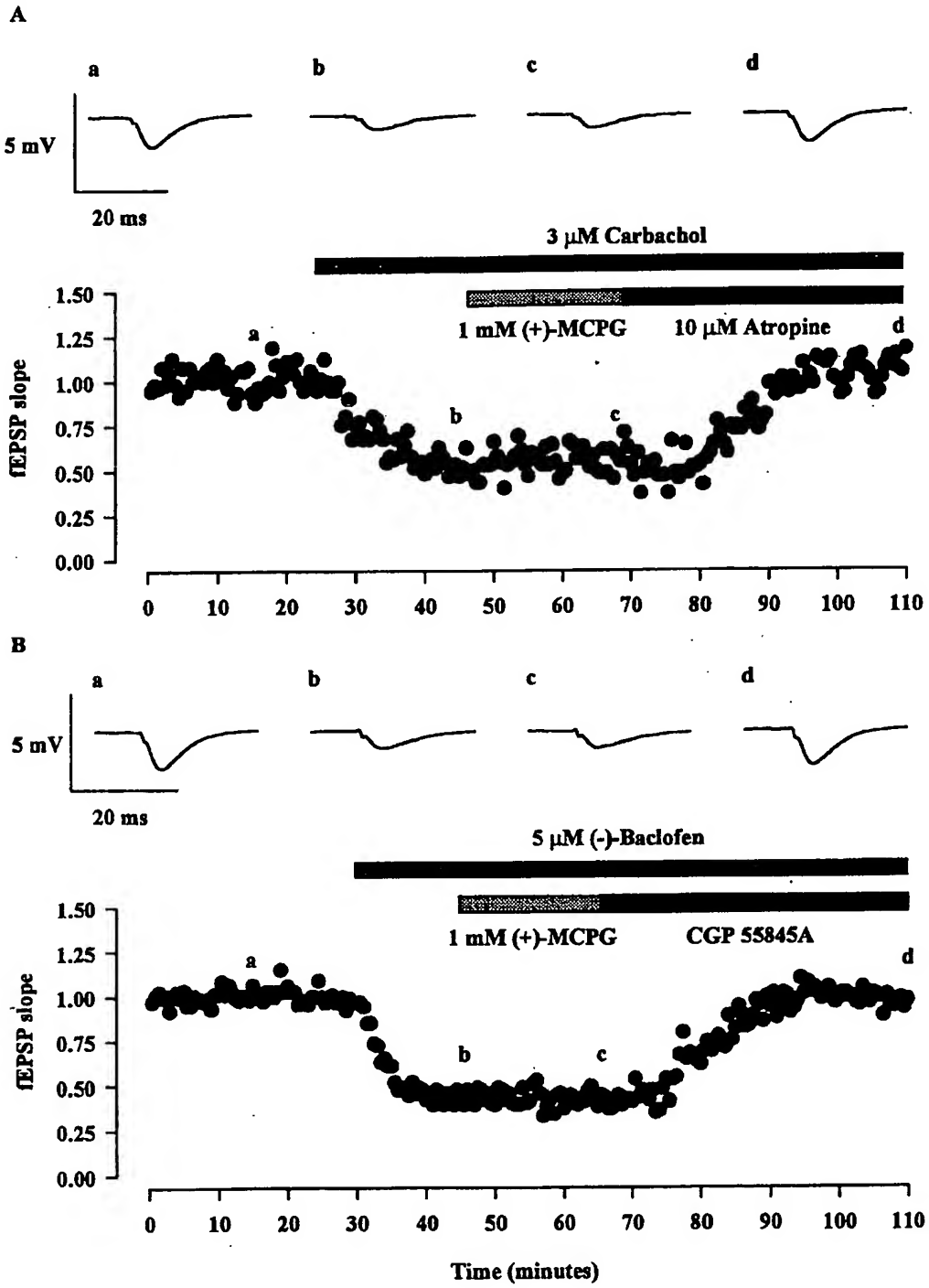

Fig. 4. Selectivity of (+)-MCPG towards mGluRs. (A) A single example showing the failure of (+)-MCPG to reverse depressions induced by carbachol. Atropine, however, reversed the depression. (B) A single example showing the failure of (+)-MCPG to reverse depressions induced by (−)-baclofen. CGP55845A (1 μM), however, reversed the depression.

was quite small but is probably an underestimate of its effectiveness as an antagonist since, in most experiments, there was incomplete recovery from the depressions induced by (1*S*,3*S*)-ACPD [Fig. 8(C)]. In contrast, the effects of L-AP4 were generally fully reversible [e.g. Fig. 5(A)].

Actions of (S)-4CPG

To complete the analysis of subgroup selective antagonists, we examined the effects of (*S*)-4CPG using both extracellular and whole-cell patch-clamp recording. The effects of (*S*)-4CPG were protocol-dependent. In field

potential recording experiments where high concentrations of agonists generated only small responses, (*S*)-4CPG (1 mM) reversed depressions induced by (1*S*,3*R*)-ACPD (50 μM; $n = 2$) and by (1*S*,3*S*)-ACPD (100 μM; $n = 4$) but had no effect on depressions induced by L-AP4 (100 μM; $n = 3$) (data not shown). In contrast, in whole-cell patch-clamp experiments where (1*S*,3*S*)-ACPD (10 μM) produced substantial *j* depressions in voltage-clamped cells, (*S*)-4CPG had no effect [Fig. 8(C)].

DISCUSSION

The present results confirm that (1*S*,3*R*)-ACPD depresses synaptic transmission at the Schaffer collateral–commissural synapse in the rat (Harvey *et al.*, 1991; Desai *et al.*, 1992). A small part of this effect could be due to the depolarizing action of (1*S*,3*R*)-ACPD. However, a large proportion of the effect is probably presynaptic (Baskys and Malenka, 1991). The finding that L-AP4 also depresses synaptic transmission is also in agreement with previous results. Since L-AP4 has no direct excitatory action, unless the preparation is firstly "primed" with, e.g. quisqualate (Robinson *et al.*, 1986) it can be assumed that the effect of L-AP4 on field EPSPs is exclusively presynaptic (Baskys and Malenka, 1991). The depressions of EPSCs by L-AP4 can be considered to be entirely presynaptic. In contrast to a previous report (Desai *et al.*, 1992), we found (1*S*,3*S*)-ACPD to have a similar effect to (1*S*,3*R*)-ACPD. Once again a part of its effect on field EPSPs could be indirect due to its depolarizing action, but its effect on EPSCs can be assumed to be entirely presynaptic. The reason why an earlier study (Desai *et al.*,

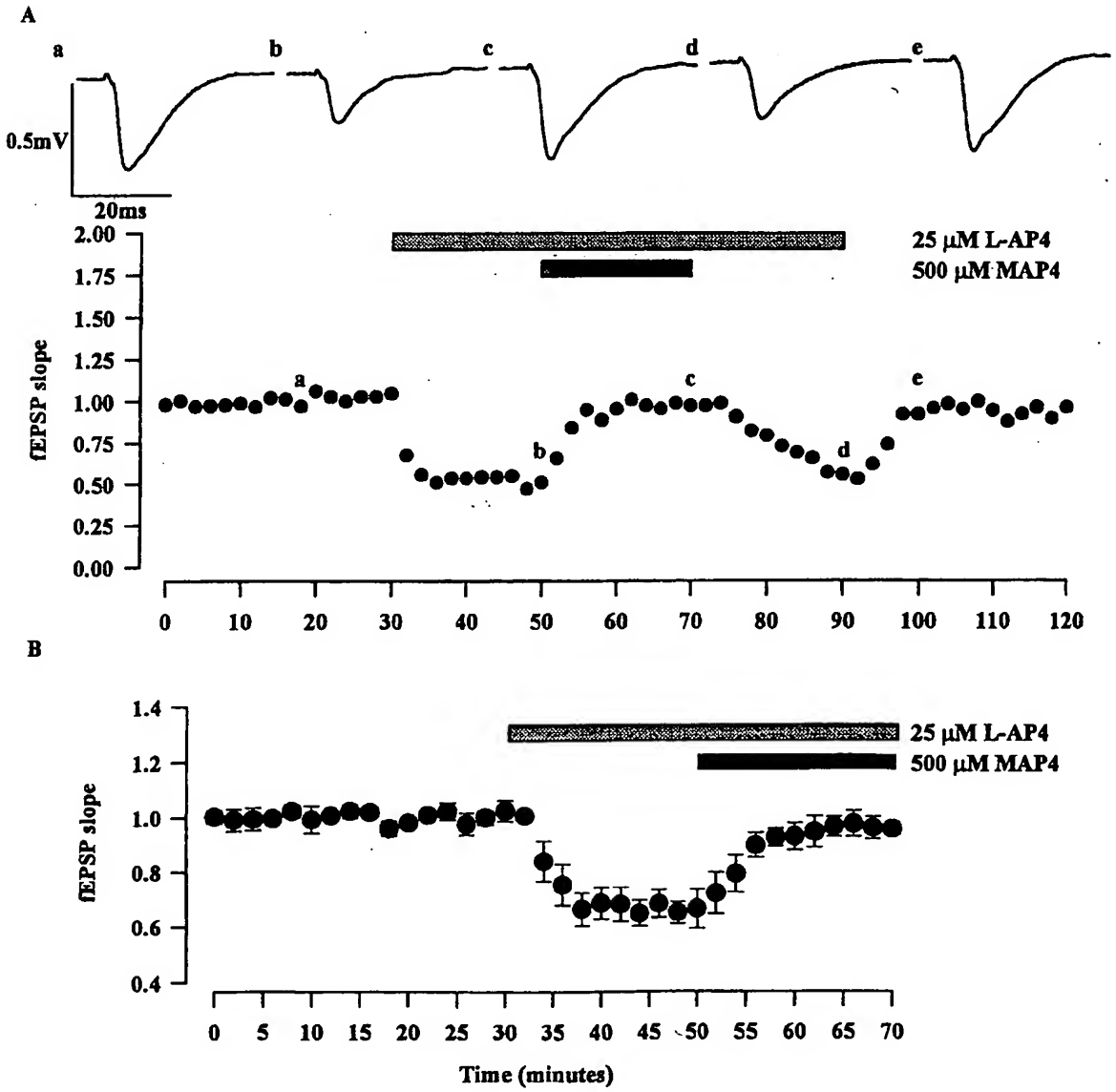

Fig. 5. MAP4 antagonizes depressions of field EPSPs induced by L-AP4. (A) Shows a single example of reversible antagonism. (B) Plots pooled data for all 4 slices using this protocol.

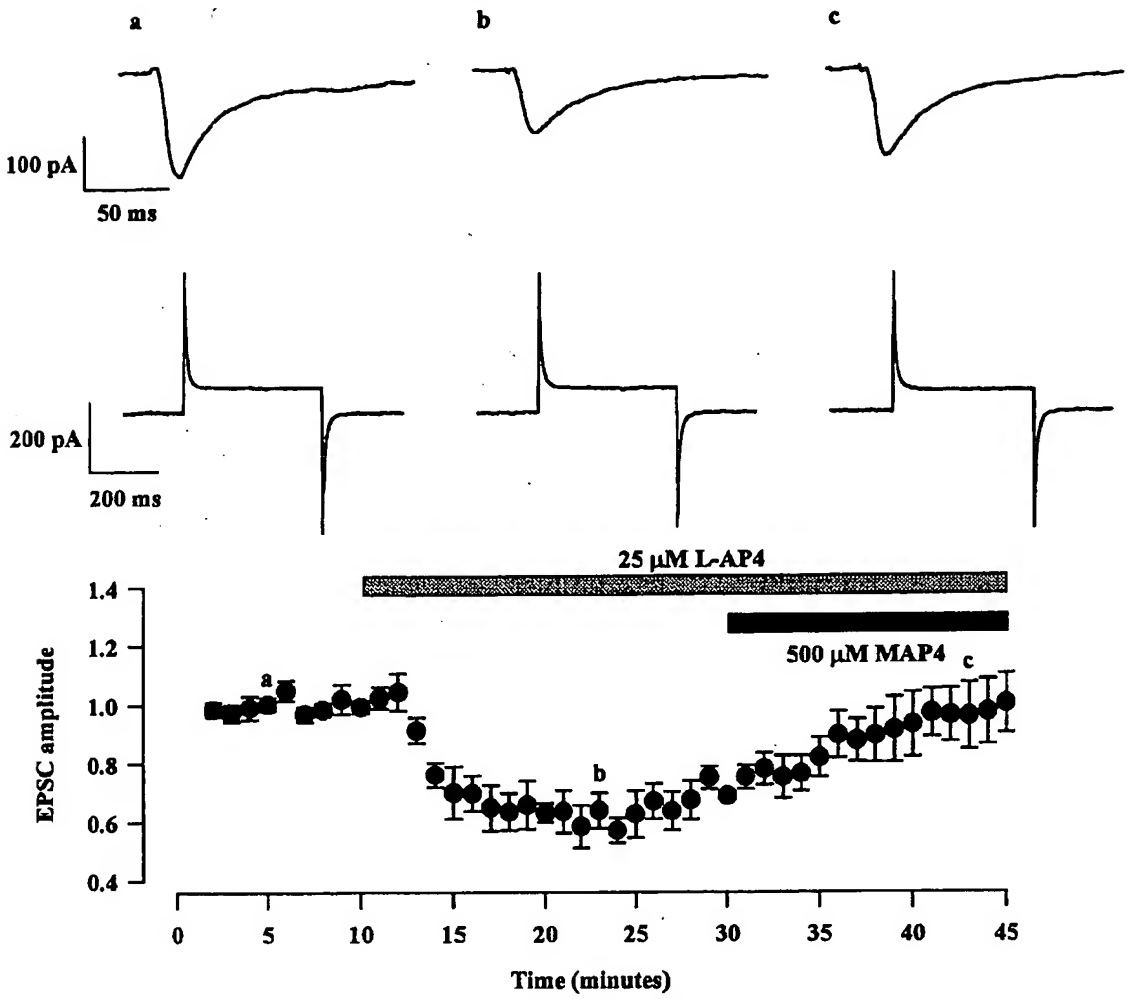

Fig. 6. MAP4 antagonizes depressions of EPSCs induced by L-AP4. The graph plots pooled data ($n = 6$) of the mean ± SEM amplitude of the EPSC plotted vs time. The traces show EPSCs (upper) and current responses to 10 mV voltage step commands (lower).

1992) failed to observe any action of (1*S*,3*S*)-ACPD is not known.

The finding that (+)-MCPG antagonized the depressant actions of (1*S*,3*R*)-ACPD extends our initial report for the Schaffer collateral–commissural synapse (Davies *et al.*, 1993). Subsequent studies in several laboratories have confirmed the effectiveness of MCPG at this site (Manzoni *et al.*, 1994; Bolshakov and Siegelbaum, 1994; Watkins and Collingridge, 1994; Selig *et al.*, 1995). However, one laboratory claimed (±) MCPG (500 μM) to be ineffective (Chinestra *et al.*, 1993) for reasons which are unclear. It is unlikely that the negative result can be attributed to the use of a 4-fold lower concentration of the effective (+) enantiomer than used in the present study, since some of the studies with positive results also used 500 μM (±)-MCPG. The present observations that (+)-MCPG does not affect the depressant actions of carbachol or baclofen, agonists for presynaptic G-protein linked cholinoceptors and GABA receptors, extends the information on the selectivity of (+)-MCPG towards mGluRs as opposed to other receptor systems (Bashir *et al.*, 1993).

MAP4 consistently reversed the effects of L-AP4. Similar antagonism of L-AP4-induced depressions has also been reported in the spinal cord (Jane *et al.*, 1994) and lateral perforant path synapse in the dentate gyrus (Bushell *et al.*, 1995). The finding that MAP4 antagonized the depressant actions of L-AP4 but not those of (1*S*,3*S*)-ACPD, while (+)-MCPG was more effective against (1*S*,3*S*)-ACPD than against L-AP4, and MCCG antagonized depressions induced by (1*S*,3*S*)-ACPD but not depressions induced by L-AP4 demonstrates the existence of two subtypes responsible for the presynaptic depressant actions of mGluR agonists at this synapse. A similar conclusion, based on antagonist studies, was made originally for the depression of monosynaptic excitation in the spinal cord (Jane *et al.*, 1994) and this principle has been extended to other synapses; inhibitory synapses in the thalamus (Salt and Eaton, 1995), the mossy fibre pathway in the hippocampus (Manzoni *et al.*,

1995) and the lateral perforant path projection in the dentate gyrus (T. J. Bushell, unpublished observations). The full subtype specificities for the antagonists used are not yet known. It is likely, however, that the effects of MCCG and MAP4 involve actions on members within subgroup II (mGluR 2 or 3) and subgroup III (mGluRs 4,6–8), respectively.

The finding that (*S*)-4CPG did not reverse depressions induced by (1*S*,3*S*)-ACPD in voltage-clamped cells is consistent with the effect of this agonist being at group II mGluRs. Indeed, (*S*)-4CPG caused a further small enhancement of the depression; an effect consistent with its agonist action at this receptor subgroup (Watkins and Collingridge, 1994). Its ability to reverse small depressions induced by higher doses of (1*S*,3*R*)- and (1*S*,3*S*)-ACPD in the extracellular experiments can be explained by an indirect effect. Thus, at these concentrations both (1*S*,3*R*)- and (1*S*,3*S*)-ACPD depolarize CA1 neurones via an action at (*S*)-4CPG-sensitive receptors (Davies *et al.*, 1995 and V. R. J. Clarke, unpublished observations); the size of the depolarizations (c. 10 mV) is sufficient to account for the small synaptic depressions (by reducing the driving force on the synaptic response). However, the possibility of presynaptic group I mGluRs, detectable under other conditions, cannot be excluded.

A paradoxical finding was that using (1*S*,3*S*)-ACPD large depressions were consistently observed in the patch-clamp experiment whereas only occasionally were large depresssions seen in the extracellular experiments. This difference was observed despite using slices obtained from the same animals for both sets of experiments, for reasons which are currently unclear. On a few occasions when large depressions of field EPSPs were observed with low concentrations of (1*S*,3*S*)-ACPD (e.g. 10 μM) the sensitivity to the slices to the group II specific agonist

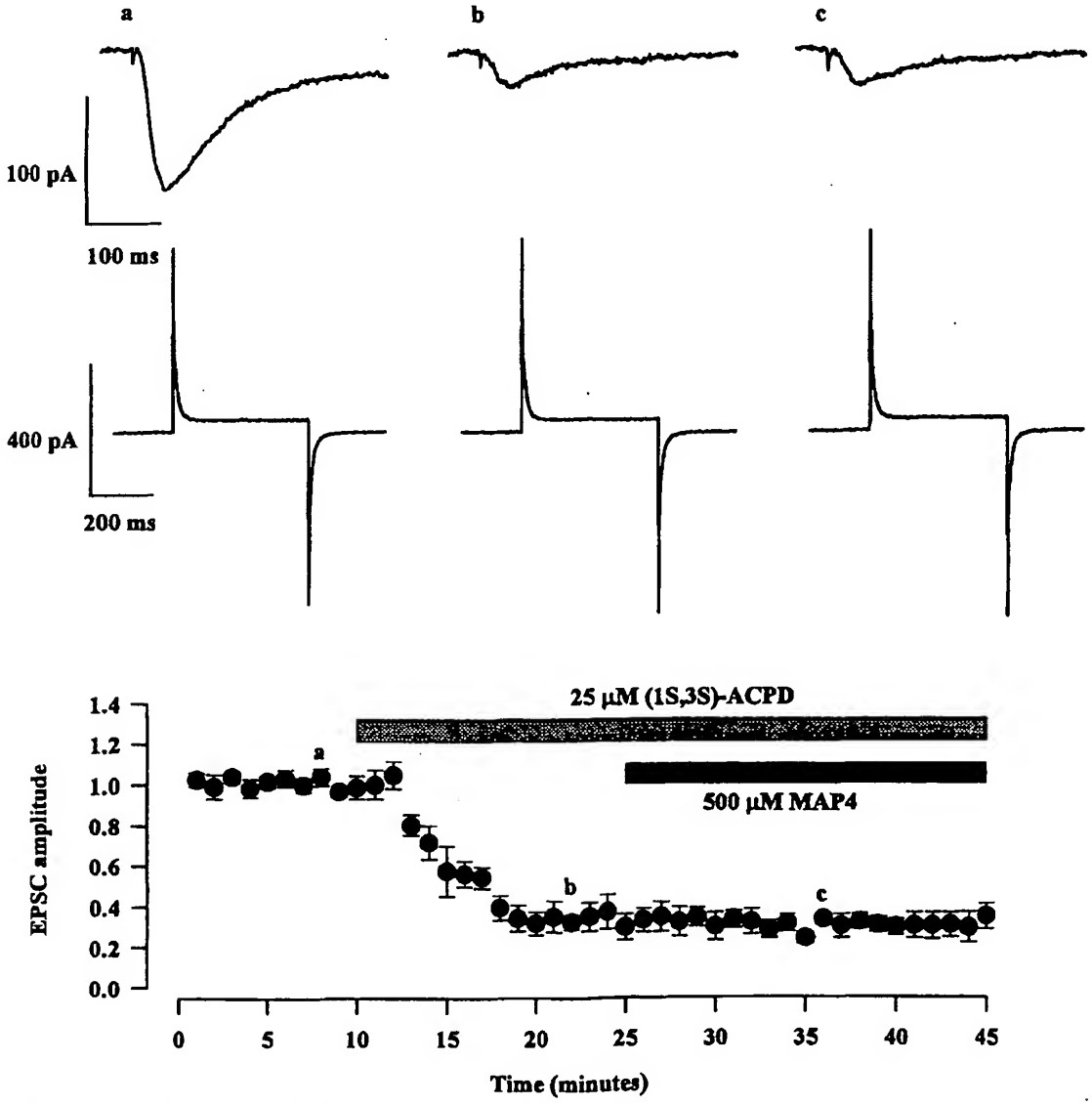

Fig. 7. MAP4 does not affect depressions of EPSCs induced by (1*S*,3*S*)-ACPD. The graph plots pooled data ($n = 6$).

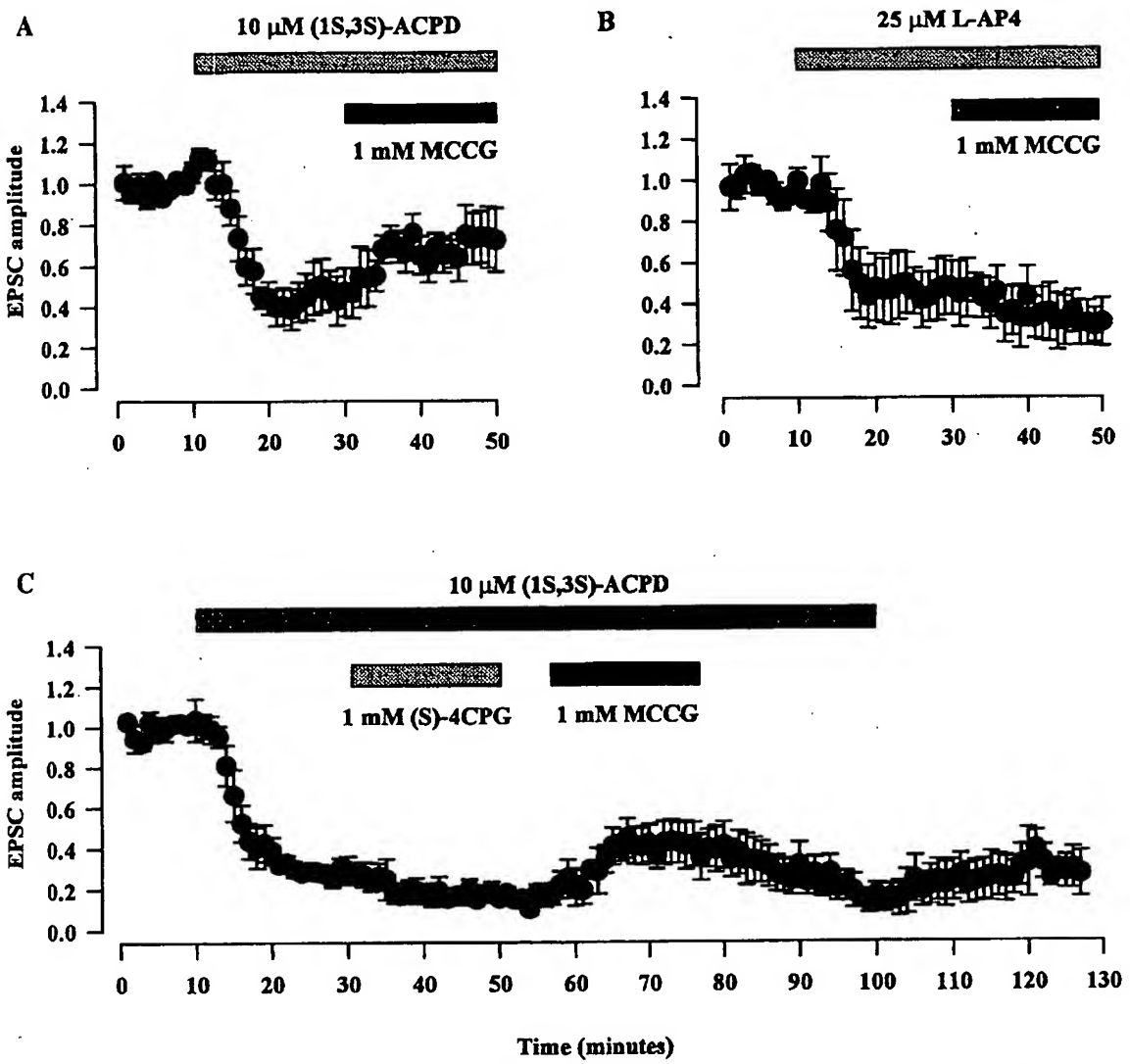

Fig. 8. MCCG reverses (1*S*,3*S*)-ACPD induced depressions. Pooled data showing the effects of MCCG on depressions induced by (A) (1*S*,3*S*)-ACPD ($n = 5$) and (B) L-AP4 ($n = 3$). In (C) are compared the effects of (*S*)-4CPG and MCCG on depressions induced by (1*S*,3*S*)-ACPD ($n = 3$). Note poor reversal of the effects of (1*S*,3*S*)-ACPD following washout of the agonist.

(2*S*,1′*R*,2′*R*,3′*R*)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV) (Ishida *et al.*, 1993) was also tested. This agent consistently depressed synaptic transmission at 1 μM (V. R. J. Clarke, unpublished observations), a concentration which is subthreshold for activating hippocampal NMDA receptors (Wilsch *et al.*, 1994).

In conclusion, the present results provide evidence for two types of mGluRs which inhibit neurotransmitter release at the Schaffer collateral–commissural synapse and which can be selectively antagonized by MCCG and MAP4, respectively.

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COMMENTARY

NOVEL FUNCTIONS FOR SUBTYPES OF METABOTROPIC GLUTAMATE RECEPTORS

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Abstract—Metabotropic or "G-protein coupled" glutamate receptors (mGluRs) were discovered and established as a new type of excitatory amino acid receptor by their unique coupling mechanism (phosphoinositide hydrolysis) and pharmacological characteristics. Recently, the cloning of mGluRs and the availability of selective pharmacological agents has greatly increased knowledge of these receptors. It is now recognized that mGluRs are a highly heterogenous family of glutamate receptors with novel molecular structure that are linked to multiple second messenger pathways. Members of this family have unique pharmacological properties and function to modulate the presynaptic release of glutamate and the postsynaptic sensitivity of the cell to glutamate excitation. New information on mGluRs is elucidating the functions of mGluR subtypes in normal and pathological aspects of neuronal transmission. Basic knowledge of the role of specific mGluRs in CNS function and pathologies will further expand in the near future. This knowledge is providing the framework for the discovery of novel pharmacological approaches to modulate excitatory amino acid neuronal transmission.

L-Glutamate is an endogenous excitatory amino acid neurotransmitter substance that contributes excitatory input into the majority of synapses in the central nervous system. Recent molecular studies have demonstrated that glutamate acts on two major classes of receptors, ionotropic glutamate receptors (iGluRs) and metabotropic glutamate receptors (mGluRs).

iGluRs are ligand-gated integral ion channels that are composed of multiple subunit proteins. Each subunit protein is characterized by a relatively large N-terminal extracellular domain with four hydrophobic membrane spanning regions and selective permeability to monvalent or divalent cations. Based on their primary sequence and their agonist sensitivity when expressed, iGluRs are classified into three types: *N*-methyl-D-aspartate (NMDA), kainate, and AMPA receptors (see Barnard and Henley, 1990; Sommer and Seeburg, 1992; Barnes and Henley, 1992).

mGluRs were initially characterized as G-protein linked (i.e. pertussin toxin sensitive) glutamate receptors that were coupled to activation of phosphoinositide hydrolysis, and thus the mobilization of intracellular calcium (Sugiyama *et al.*, 1987). These receptors were demonstrated to exist in various tissues including cultured neurons (Sladeczek *et al.*, 1985), brain slices (Nicoletti *et al.*, 1986), glia (Pearce *et al.*, 1986), and retinal cells (Osborne, 1990) by the ability of various non-selective mGluR agonists (i.e. quisqualate, ibotenate) to increase phosphoinositide hydrolysis. Later it was found that the rigid glutamate analog (±)*trans*-1-aminocyclopentane-1,3-dicarboxylic acid (*trans*-ACPD) (Palmer *et al.*, 1989; Desai and Conn, 1990) or its 1S,3R- isomer (1S,3R-ACPD) (Irving *et al.*, 1990; Schoepp *et al.*, 1991) activates phosphoinositide hydrolysis in CNS tissues at concentrations with no effect on iGluRs. The availability of this first selective mGluR agonist allowed investigators to begin probing the cellular consequences of selectively activating mGluRs (see Schoepp and Conn, 1993).

It is now known that 1S,3R-ACPD acts on multiple mGluRs, and this leads to a variety of biochemical responses in the target cell (see Table 1; Schoepp and Conn, 1993). mGluR activation with 1S,3R-ACPD has also been shown to modulate the excitability of a number of cell types through a variety of mechanisms (see Table 1). For example, 1S,3R-ACPD inhibits slow afterhyperpolarization (I_{AHP}) that is responsible for accomodation, and this results in repetitive firing of these neurons (Stratton *et al.*, 1989; Charpak and

Table 1. Biochemical and cellular consequences of *in situ* mGluR activation[a]

(1) Changes in second messenger systems	
↑ Phospholipase C	↑ Phospholipase D
↓ cAMP	↑ cAMP
↑ Arachidonic acid	↑ cGMP
(2) Modulation of ion channel currents (↓)	
↓ I_{AHP}	↓ I_M
↓ I_K (Ca^{2+}-independent)	↑ I_K(CA^{2+}-dependent)
↓ $I_{Ca^{2+}}$-(*N*-type)	↑ $I_{Ca^{2+}}$-(L-type)
(3) Modulation of ligand gating ionotropic receptors	
↑ I_{NMDA}	↑ I_{AMPA}
↓ $I_{Muscimol}$	
(4) Modulation of neurotransmitter release	
↓ Glutamate	↑ Glutamate (PI linked)
↓ GABA	↑ Dopamine
(5) Modulation of synaptic plasticity	
↑ LTP	↑ LTD

[a] Also see Conn and Desai (1991) and Schoepp and Conn (1993).

Gahwiler, 1991). 1S,3R-ACPD-sensitive mGluRs also mediate inhibition of voltage sensitive calcium channels in some neurons (Swartz and Bean, 1992; Trombley and Westbrook, 1992; Sayer *et al.*, 1992), facilitate of iGluR-mediated currents (Aniksztejn *et al.*, 1992; Kelso *et al.*, 1992; Collins, 1993; Bleakman *et al.*, 1992), and participate in long-term changes in neuronal excitability such as long-term-potentiation (LTP) (Ito and Sugiyama, 1991; Aniksztejn *et al.*, 1992; Zheng and Gallagher, 1992; Izumi *et al.*, 1991; Behnisch and Reyman, 1993; Bashir *et al.*, 1993) and long-term-depression (LTD) (Linden *et al.*, 1991; Liu *et al.*, 1993). However, an association between these functional effects of 1S,3R-ACPD and the activation of phosphoinositide hydrolysis or alterations of cAMP formation have been difficult to establish (see Schoepp and Conn, 1993). It is now known that mGluR subtypes fall into three groups based on their pharmacological characteristics and second messenger coupling (see Table 2, and text below). With the knowledge gained by the cloning and characterization of mGluR subtypes, the molecular basis for these effects of 1S,3R-ACPD and other mGluR agonists are now being revealed. This article focuses on select aspects of recent structural and pharmacological information which has enhanced understanding of the cellular mechanisms associated with activation of mGluR subtypes. More comprehensive reviews on mGluR molecular structure and the cellular consequences of mGluR activation have been published elsewhere (see Schoepp *et al.*, 1990; Conn and Desai, 1991; Baskys, 1992; Barnes and Henley, 1992; Mayer and Miller, 1990; Miller, 1991; Nakanishi, 1992; Schoepp and Conn, 1993).

CLONING AND CHARACTERIZATION OF A HETEROGENOUS FAMILY OF mGluRs

The *xenopus* oocyte offers a sensitive system which can be used to express and study phosphoinositide coupled membrane receptors. Early work showed that mGluRs could be expressed following rat brain mRNA injection in the *xenopus* oocyte by their coupling through a pertussis toxin sensitive G-protein to phospholipase C (Sugiyama *et al.*, 1987, 1989). Inositol trisphosphate, which is formed following mGluR activation of phospholipase C, mobilizes intracellular

Table 2. Pharmacology of cloned mGluRs[a]

Receptor	Second messenger	Pharmacological characteristics
Group 1[b]		
mGluR1α	↑ Phosphoinositide hydrolysis	Potently activated by quisqualate and 1S,3R-ACPD
mGluR1β		Insensitive to L-AP4
mGluR1c	Postsynaptic	
mGluR5		
mGluR5b		
Group 2[c]		
mGluR2	↓ cAMP	Potently activated by L-CCG-I and 1S,3R-ACPD
mGluR3	Presynaptic	Insensitive to quisqualate and L-AP4
		Sensitive to pertussis toxin (PTX) inhibition
Group 3[d]		
mGluR4	↓ cAMP	Potently activated by L-AP4 and L-SOP
mGluR6		Insensitive to quisqualate and 1S,3R-ACPD
mGluR7		Sensitive to pertussis toxin (PTX) inhibition

[a] See Nakanishi (1992). [b] Primary references: mGluR1α, Masu *et al.* (1991), Houamed *et al.* (1991); mGluR1β, Tanabe *et al.* (1992); mGluR1c, Pin *et al.* (1992); mGluR5, Abe *et al.* (1992); mGluR5b, Minakami *et al.* (1993). [c] Primary references: mGluR2, Tanabe *et al.* (1992); mGluR3, Tanabe *et al.* (1993). [d] Primary references: mGluR4, Tanabe *et al.* (1993); mGluR6, Nakajima *et al.* (1993), mGluR7, Saugstad *et al.* (1993), Okamoto *et al.* (1994).

calcium, and this could be detected in the *xenopus* oocyte in a highly sensitive manner by the measurement of calcium-activated chloride currents. Using this system, two independent groups expression cloned a phosphoinositide coupled mGluR (now termed mGluR1α) from a rat cerebellar library (Masu *et al.*, 1991; Houamed *et al.*, 1991). The molecular structure of mGluR1α makes it a highly unique G-protein coupled receptor. Like other G-protein coupled receptors that have been cloned, mGluR1α possesses eight hydrophobic regions that likely correspond to a N-terminus signal peptide and seven membrane spanning motifs. However, the primary sequence and size of mGluR1α clearly distinguish it from other G-protein linked receptors. Rat mGluR1α has no significant sequence homology with non-glutamate G-protein-coupled receptors that have been cloned. In addition, mGluR1α is quite large by comparison, with a total size of 1199 amino acids and a molecular size of 133,229 daltons. The size of mGluR1α is remarkable considering that it functions to bind and transduce the effects of a small molecule such as glutamate.

The expression cloning of mGluR1α has rapidly lead to the discovery of a heterogenous family of mGluRs that appear to be coupled to multiple effectors *in situ*. At least seven different mGluR subtypes (mGluR1–mGluR7), along with various alternate splice versions of mGluR1 and mGluR5 (see Table 2), have been cloned. These mGluRs fall into three groups based on their degree of sequence homology and pharmacology. Members within each group have about 70% homology with each other (as opposed to 40% homology between groups). The higher homology within each mGluR group is associated with the expression of the same transduction mechanism and similar agonist pharmacology. These groups include: (1) mGluR1 (α, β, and c forms) and mGluR5 (a and b forms) which are coupled to increased phosphoinositide hydrolysis and are potently activated by quisqualate and with lower potency, *trans*-ACPD; (2) mGluR2 and mGluR3, which are negatively linked to adenylyl cyclase, are most potently activated by *trans*-ACPD, but are insensitive to quisqualate; and (3) mGluR4, mGluR6, and mGluR7, which are negatively coupled to adenylyl cyclase, are potently activated by L-2-amino-4-phosphonobutyrate (L-AP4) and L-serine-O-phosphate (L-SOP), but are relatively insensitive to *trans*-ACPD and quisqualate. Each of these mGluRs has a unique distribution in the CNS and retina, and this likely reflects a diversity of functions in normal and pathological processes (see Nakanishi, 1992).

With the cloning of mGluRs, the functional domains of mGluRs that are responsible for ligand binding and G-protein interaction can now be explored. mGluRs differ from other G-protein coupled receptors by their large size, including the presence of a large N-terminus extracellular domain, and they have no significant sequence homology with other G-protein coupled receptors, even in the transmembrane regions of the receptor. The unique molecular structure of mGluRs thus renders structural models of other G-protein receptors non-applicable, and suggests that the functional domains for mGluRs that are responsible for ligand binding and G-protein interaction may be distinct. The ligand binding domain for other G-protein coupled receptors (i.e. dopamine, serotonin, muscarinic) has been suggested to be in the transmembrane region (see Findlay and Eliopoulos, 1990; Hibert *et al.*, 1991). This does not appear to be the case for mGluRs. A recent study (O'Hara *et al.*, 1993) suggests that the mGluR ligand binding domain may be in the amino-terminus extracellular domain of the protein. In their study they showed that the mGluR extracellular domain is similar in structure to bacterial periplasmic amino acid binding proteins. The mGluR ligand binding site was predicted to be present in the extracellular amino-terminus domain based on regions of similarity between these proteins. Specifically, the side chains of mGluR1 amino acids serine-165 and threonine-188 were hypothesized to hydrogen bind to the α-amino acid backbone of the ligand (glutamate). In further support of this hypothesis, mutants of mGluR1 with non-polar amino acid substitutions at these positions (alanine-165 and/or alanine-188) were shown to reduce agonist (quisqualate and glutamate) potency in activating phosphoinositide hydrolysis and affinity in displacing ^{3}H-glutamate binding.

Consistent with the N-terminus region possessing the glutamate recognition site for mGluRs, it has recently been reported that the pharmacological characteristics of an mGluR subtype, but not its G-protein/second messenger specificity, can be altered by the amino acid composition of the extracellular domain. In the study of Takahashi *et al.* (1993) chimeric mGluRs were prepared, exchanging N-terminus regions of mGluR1α (a quisqualate-sensitive, phosphoinositide linked mGluR), with sequences from mGluR2 (a quisqualate-insensitive negatively-coupled cAMP-linked mGluR). Such chimerics retained mGluR1α-like coupling to phosphoinositide hydrolysis, as measured by glutamate-induced currents in the *xenopus* oocytes. However, the chimeric mGluR1α receptors lost their wild-type quisqualate

sensitivity, and gained sensitivity to the agonists *trans*-ACPD and (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine (DCG-IV), which are more potent at wild type mGluR2 receptors compared to mGluR1α. These data clearly show that the pharmacological differences between mGluR subtypes are confered in the amino acid composition of the extracellular region of the mGluR. Information such as this may be useful to create 3-D protein structural models of the mGluR ligand recognition sites. Such molecular models might be used to design highly specific ligands for mGluR subtypes in the future.

CELLULAR FUNCTIONS OF PHOSPHOINOSITIDE LINKED mGluRs

The phosphoinositide coupled mGluRs (mGluR1 and mGluR5) are highly sensitive to quisqualate and can be selectively activated by 1S,3R-ACPD (see Table 2). Thus, quisqualate sensitivity at low micromolar concentrations, along with sensitivity to the more selective agonist 1S,3R-ACPD, would be indicative of the specific involvement of mGluR1 and/or mGluR5 subtypes, and possibly the phosphoinositide second messenger system, in mediating a particular *in situ* mGluR response. Consistent with this, both quisqualate and 1S,3R-ACPD have been demonstrated to mobilize intracellular calcium stores in glia (Glaum *et al.*, 1990; Ahmed *et al.*, 1990) and in dendritic processes and the cell soma of neurons (Murphy and Miller, 1988; Irving *et al.*, 1992; Vranesic *et al.*, 1991), likely via inositol-1,4,5-triphosphate induced calcium release. The activation of phosphoinositide linked mGluRs also increases diacylglycerol formation and thus enhances protein kinase C activity (Manzoni *et al.*, 1990).

A variety of cellular responses that exhibit both quisqualate and 1S,3R-ACPD sensitivity, but are insensitive to blockade by iontropic glutamate receptor antagonists, have been reported. These responses have a pharmacology consistent with the selective activation mGluR1 and/or mGluR5. For example quisqualate and *trans*-ACPD mobilize intracellular calcium and thus activate a large conductance calcium-dependent potassium channel in cultured cerebellar granule cells (Fagni *et al.*, 1991). This would reduce the excitability of these cells to other excitatory inputs such as those from ionotropic glutamate receptor activation. Quisqualate and 1S,3R-ACPD-sensitive mGluRs have also been shown to enhance NMDA receptor mediated currents in hippocampal neurons (Aniksztejn *et al.*, 1991) or in the *xenopus* oocyte (Kelso *et al.*, 1992) via the activation of protein kinase C. However, in the study of Harvey and Collingridge (1993) enhancement of NMDA currents by 1S,3R-ACPD was not blocked by inhibitors of protein kinase C or depletion of intracellular calcium by thapsigargin (Harvey and Collingridge, 1993). Regardless of the underlying mechanism(s), mGluR-mediated enhancement of ionotropic glutamate receptor activation may underly long-term potentiation (Aniksztejn *et al.*, 1992; Bashir *et al.*, 1993) or the induction of seizure states or excitoxicity in animals (Sacaan and Schoepp, 1992) (see Fig. 1).

Quisqualate activates phosphoinositide linked mGluRs at presynaptic sites as well, since it will mobilize intracellular calcium in synaptosomes from the rat cerebral cortex (Adamson *et al.*, 1990). Phosphoinositide linked mGluRs appear to modulate the release of glutamate from presynaptic nerve endings. Both quisqualate and 1S,3R-ACPD enhance the calcium-dependent release of glutamate from synaptosomes of the rat cerebral cortex (Herrero *et al.*, 1992a). Activation of mGluRs in synapsosomes is also linked to a rapid and transient increase in diacylglycerol levels and to subsequent activation of protein kinase C (Sanchez-Prieto *et al.*, 1993). The enhanced release of glutamate which is linked to mGluR activation can be mimicked by phorbol esters that activate protein kinase C, and both effects require the presence of low concentrations of arachidonic acid (Herrero *et al.*, 1992a, b). Activation of postsynaptic ionotropic glutamate receptors (NMDA and AMPA) leads to arachidonic acid formation (Dumuis *et al.*, 1988, 1990) and this arachidonic acid might serve as a retrograde second messenger to facilitate mGluR-mediated positive feedback release of transmitter glutamate (see Nicholls, 1992). Since this mGluR-mediated presynpatic positive feedback mechanism would appear to work in concert with postsynaptic events such as NMDA receptor activation, it may have a physiological role in the maintenance of long-term potentiation of synaptic responses or in pathological conditions of enhanced glutamatergic transmission (i.e. seizures and excitotoxicity) (see Fig. 1). A number of different lines of evidence using the mGluR antagonist compound L-2-amino-3-phosphonopropionic acid (L-AP3) support this hypothesis. L-AP3 has been reported to antagonize 1S,3R-ACPD- and quisqualate-induced phosphoinositide hydrolysis in slices of the rat hippocampus or cerebral cortex (see Schoepp *et al.*, 1990) or in mGluR1α expressing cells (Houamed *et al.*, 1991), antagonize the mGluR-mediated enhanced release of glutamate from synaptosomes (Herrero *et al.*, 1992a, b), block late synaptic long-term potentiation in the hippocampus (Behnisch

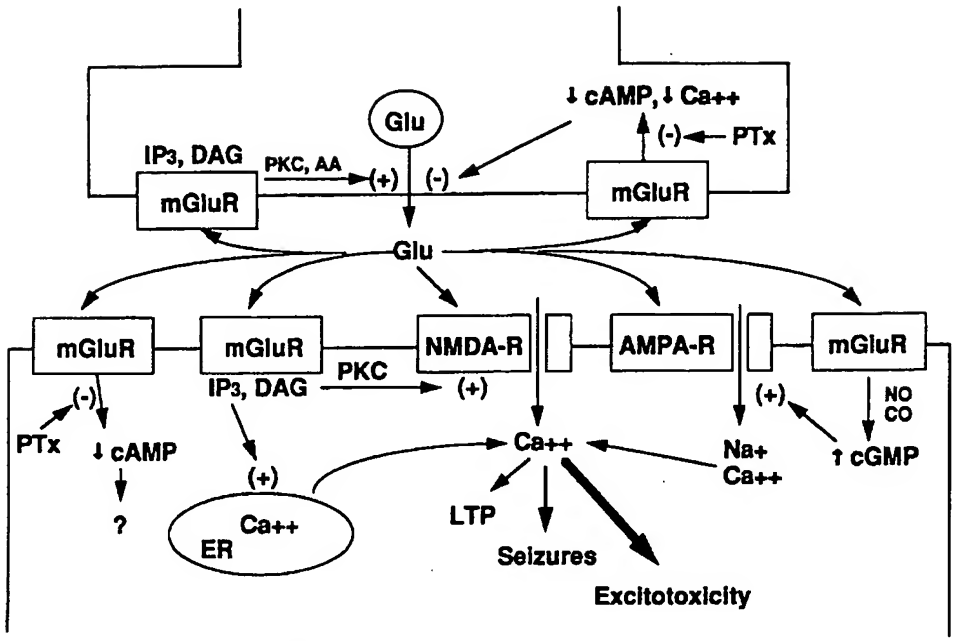

Fig. 1. Mechanisms for mGluR mediated modulation of glutamatergic neuronal transmission. The presynaptic release of glutamate is regulated by both positive and negative feedback mechanisms that involve phosphoionositide-coupled or negatively linked cAMP coupled mGluR autoreceptors, respectively. The specific mGluR subtypes that are localized presynaptically and control glutamate release remain to be determined, and other effectors such as modulation of presynaptic calcium channels may be involved. For negative feedback mGluR autoreceptors, both L-AP4-sensitive and 1S,3R-ACPD-sensitive/L-AP4 insensitive subtypes have been demonstrated. Positive feedback mGluR autoreceptors are linked to phosphoionositide hydrolysis and transduce this effect by the combined effects of diacylglycerol (DAG) and arachidonic acid (AA) to increase protein kinase C (PKC) activity. Postsynaptic quisqualate and 1S,3R-ACPD sensitive mGluRs have been demonstrated to modulate ionotropic glutamate receptor currents, including NMDA and AMPA receptors. In the case of NMDA receptors this may involve PKC mediated phosphorylation subsequent to activation of phosphoionositide coupled mGluRs. AMPA receptor currents are also enhanced by mGluR activation by a process that involves a diffusable second messenger such as carbon monoxide (CO) which can activate guanyl cyclase and increase cGMP. Through these mechanisms pre- and postsynaptic mGluRs function to modulate the excitability of the postsynaptic neurons which are receptive to transmitter glutamate. These modulatory mechanisms may play a physiological role in plasticity phenomenon such as long-term synaptic potentiation (LTP), and pathological states that involve excessive or inappropriate glutamatergic neuronal transmission such as seizure states and the expression of glutamate excitotoxicity.

et al., 1991), block limbic seizures induced by intracerebral administration of 1S,3R-ACPD (Tizzano *et al.*, 1993), and block audiogenic seizures in DBA/2 mice (Klitgaard and Jackson, 1993). Thus, more potent and systemically active antagonist agents which act at the quisqualate- and 1S,3R-ACPD-sensitive mGluR subtypes may offer a novel therapeutic approach to treat epilepsy and various neurodegenerative conditions involving enhanced glutamate transmission and glutamate excitotoxicity.

CELLULAR FUNCTIONS OF cAMP LINKED mGluRs

The discovery and characterization of multiple mGluRs has rapidly expanded understanding about the functions of glutamate receptors in the brain. For example, the second of these to be cloned, mGluR2, was described as a novel mGluR with 46% sequence homology to mGluR1α. Interestingly, mGluR2 was found to be negatively coupled to cAMP formation when expressed in non-neuronal cells (Tanabe *et al.*, 1992). Agonists including the selective mGluR compound *trans*-ACPD and glutamate potently inhibited forskolin-stimulated cAMP formation in CHO cells expressing mGluR2. This effect of these agonists was highly sensitive to inhibition by pertussis toxin. The discovery and characterization of mGluR2 in this manner provided the first convincing evidence that glutamate receptors which are directly linked to

adenylate cyclase via G-proteins may exist *in situ*. In support of this, inhibition of forskolin-stimulated cAMP formation by *trans*- or 1S,3R-ACPD has been demonstrated in neuronal tissues such as brain slices and cultured neurons (Schoepp *et al.*, 1992; Cartmell *et al.*, 1992; Prezeau *et al.*, 1992). Thus, it was recognized that the cellular consequences following mGluR activation with 1S,3R-ACPD could be a result of altered cAMP levels, rather than being linked to the more established *in situ* changes in phosphoinositide hydrolysis.

The availability of cloned mGluRs has facilitated the discovery of more selective pharmacological tools to further facilitate understanding of the specific cellular functions of *in situ* mGluR subtypes. For example, the striatum is a region of the brain which is enriched in glutamatergic afferents arising from cortical–striatal neuronal pathway. In electrophysiological studies using striatal neurons, the mGluR agonist *trans*-ACPD, which acts on multiple mGluRs (see Table 2). depresses synaptic transmission presumably by acting on a presynaptic site to decrease glutamate release (Lovinger, 1991). In subsequent work it was reported that this effect of *trans*-ACPD was not mimicked by L-AP4 (Lovinger *et al.*, 1993). Thus, a *trans*-ACPD-sensitive but L-AP4 insensitive mGluR subtype (such as mGluR2) appears responsible for this presynaptic mGluR effect. Using mGluR2 expressing non-neuronal cells it has been demonstrated that mGluR2 receptors can be selectively activated with (1S,3S,4S)-(carboxycyclopropyl)glycine (L-CCG-I) ($EC_{50} = 0.3$ μM) (Hayashi *et al.*, 1992). L-CCG-I has much less affinity for quisqualate-sensitive phosphoinositide linked mGluRs, or L-AP4 sensitive cAMP linked mGluRs (Hayashi *et al.*, 1992). Thus, L-CCG-I appears useful to probe the cellular functions of mGluR2 activation *in situ*. Consistent with a role for mGluR2 in the regulation of glutamate release in the striatum, L-CCG-I inhibits the depolarization-evoked release of ^{3}H-D-aspartate from slices of the rat striatum (Lombardi *et al.*, 1993). Inhibition of release was demonstrated at concentrations of L-CCG-I that inhibit forskolin-stimulated cAMP formation, but have no effects on phosphoinositide hydrolysis. Consistent with this, other negatively coupled cAMP receptors (i.e. α-adrenergic) have also been linked to modulation of exocytosis. However, it should be noted that the effect of these receptors on exocytosis is not necessarily mediated by their effects on cyclic AMP formation (see Limbird, 1988). Thus, glutamate autoreceptors that are negatively coupled to cAMP formation may be present on glutamatergic nerve terminals in the rat striatum. These mGluRs may act by this transduction mechanism or another (i.e. inhibition of calcium channels) to inhibit glutamate release. The availability of specific mGluR antibodies could ultimately be used to verify a presynaptic localization of specific mGluR proteins in different areas of the brain. The presence of 1S,3R-ACPD sensitive/L-AP4 insensitive glutamate receptor that functions to limit the further release of glutamate by an autoreceptor mechanism offers an additional strategy for the discovery of drugs to control pathological conditions of enhanced glutamatergic transmission in selected synapses where these receptors are present (see Fig. 1).

The role of postsynaptic or even glial mGluRs that are negatively linked to cAMP formation remains to be investigated. Presumably, such receptors would modulate cell excitability by decreasing cAMP-dependent protein kinase activity in the cell. Activators of adenylate cyclase such as forskolin enhance glutamate and kainate induced currents in hippocampal neurons (Greengard *et al.*, 1991). Furthermore, inhibition of cAMP-dependent protein kinase activity with competitive inhibitors of this enzyme inhibits kainate induced currents in neurons (Wang *et al.*, 1991). Since kainate receptors are regulated by cAMP-dependent protein kinases and mGluRs control kinase activity, modulatory iGluR/mGluR interactions are plausible. Hypothetically, decreases in cellular cAMP levels induced by activation of postsynaptic receptors such as mGluR2 or mGluR3 could lower kainate induced synaptic currents and thus effectively decrease the excitability of postsynaptic cells where these receptors are co-localized (see Fig. 1). The availability of selective agonists such as L-CCG-I or DCG-IV for the group two negatively coupled cAMP linked mGluRs will allow further exploration of specific biochemical events that underly mGluR/iGluR interactions.

Cyclic AMP coupled mGluRs may also function to modulate neuronal excitability by altering transmission at inhibitory synapses. In the accessory olfactory bulb of the rat the mGluR2 selective agonist DCG-IV reduces GABA-mediated inhibitory postsynaptic currents (Hayashi *et al.*, 1993). mGluR2 is localized to granule cell dendrites in this region (Hayashi *et al.*, 1993) which form dendrodendric synapses with excitatory mitral cells. Thus, this effect of the mGluR2 selective agonist DCG-IV may reflect a mechanism to remove GABA-mediated inhibitory influences on the excitatory mitral cells and enhance olfactory sensory transmission.

L-AP4-sensitive presynaptic glutamate receptors that control glutamate release were initially described

by Koerner and Cotman (1981). They demonstrated that micromolar concentrations of L-AP4 will inhibit perforant path synaptic transmission from the entorhinal cortex to the dentate gyrus in the rat. This initial finding and other observations of L-AP4-induced supression of neuronal transmission in pathways such as the lateral olfactory tract in the rat (Hori *et al.*, 1982; Collins, 1982), spinal cord responses in the rat (Davies and Watkins, 1982), and mossy fiber inputs to CA3 regions of the hippocampus in the guinea pig (Yamamoto *et al.*, 1983; Lanthorn *et al.*, 1984) were the basis for the existence of a somewhat enigmatic "AP4" glutamate receptor subtype (see Monaghan *et al.*, 1989). For a number of years the cellular/molecular basis for classifying this receptor as either "ionotropic" or "metabotropic" was not clear. Likewise, L-AP4 sensitive receptors have been characterized in the retina (Slaughter and Miller, 1981), where this compound mimics the effects of glutamate by hyperpolarizing the ON-bipolar cells (Slaughter and Miller, 1981; Neal *et al.*, 1981; Miller and Slaughter, 1986). The recent cloning of at least three different L-AP4-sensitive mGluRs (see Table 2) has now indicated that "L-AP4" sensitive glutamate receptors are G-protein linked "metabotropic" receptors. Of the L-AP4-sensitive mGluRs, the mGluR4 receptor is a candidate for a presynaptic autoreceptor that functions at perforant path synapses to decrease glutamate release. Message for mGluR4 is highly expressed in the entorhinal cortex (Thomsen *et al.*, 1992) and L-AP4 and L-serine-O-phosphate (L-SOP), two compounds which suppress synaptic transmission in this pathway (Ganong and Cotman, 1982), are potent agonists in inhibiting cAMP formation in mGluR4 expressing non-neuronal cells (Kristensen *et al.*, 1993; Thomsen *et al.*, 1992; Tanabe *et al.*, 1993).

OTHER mGluR SECOND MESSENGER SYSTEMS

Interestingly, the expression of mGluR6 is restricted to the retina (Nakajima *et al.*, 1993). When expressed in CHO cells, mGluR6 (like mGluR4 and mGluR7) can be potently activated by L-AP4 and L-SOP. For mGluR4, mGluR6, and mGluR7 this is detected by inhibition of forskolin-stimulated cAMP formation. However, L-AP4 receptors in the retina which function to hyperpolarize ON-bipolar cells might do so by stimulation of cGMP phosphodiesterase in a G-protein dependent manner (see Nawy and Jahr, 1990, 1991; Shiells and Falk, 1992). It is possible L-AP4-sensitive retinal receptors such as mGluR6 are coupled to cGMP phosphodiesterase in its *in situ* environment (see Nakajima *et al.*, 1993). Thus, negative coupling of mGluR6 to adenylate cyclase in the CHO cell may be an example of non-physiological coupling in the absence of the native *in situ* effector for the receptor.

Activation of *in situ* mGluRs in some tissues also suggest the direct or indirect coupling to other novel second messengers. In the nucleus tractus solitarius (NTS) of the rat, 1S,3R-ACPD enhances AMPA-induced currents and suppresses $GABA_A$ currents induced by muscimol. These mGluR effects of 1S,3R-ACPD are not linked to alteration in adenylate cyclase or phospholipase C, but appear to involve the activation of soluble guanylate cyclase (Glaum and Miller, 1993a). Interestingly, the mGluR mediated activation of guanylate cyclase in this system involves the novel diffusable second messenger carbon monoxide. 1S,3R-ACPD-induced responses in the NTS are blocked by Zn-protoporphyrin-IX, a heme oxygenase inhibitor that would inhibit the production of carbon monoxide (Glaum and Miller, 1993b). Nitric oxide can also activate guanylate cyclase, but it does not appear to be involved in the NTS response to 1S,3R-ACPD since the nitric oxide synthase inhibitor L-ω-nitroarginine had no effect. However, in rat cerebellar slices 1S,3R-ACPD increases cGMP levels through a nitric oxide dependent mechanism (Okada, 1992).

A single mGluR receptor protein can also cross-talk with multiple second messengers in the same cell. For example, when rat mGluR1α was expressed in CHO cells it was shown that quisqualate activation lead to enhanced phosphoionositide hydrolysis, enhanced cAMP formation, and increased arachidonic acid release (Aramori and Nakanishi, 1992). The coupling of a single mGluR to these multiple effectors (phospholipase C, adenylate cyclase, and phospholipase A_2, respectively) in the same cell appears to involve different G-proteins, since each response was differentially affected by pertussis toxin. Interestingly, when mGluR1α was expressed in another cell (baby hamster kidney, BHK) activation by quisqualate enhanced phosphoinositide hydrolysis but did not appreciably increase cAMP formation (Thomsen *et al.*, 1993). Thus, the *in situ* environment, possibly the stoichiometry of mGluRs, G-proteins, and their effectors in a particular cells type may be an important factor in determining ultimate mGluR cellular functions.

In situ mGluRs activation has also been associated with the activation of phospholipase D (Boss and Conn, 1992; Holler *et al.*, 1993), enhanced cAMP formation through the potentiation of other receptors (i.e. adenosine) that are directly coupled to adenylyl

cyclase via G_s (Casabona *et al.*, 1992; Winder and Conn, 1992, 1993; Alexander *et al.*, 1992), and possibly the direct coupling to ion (i.e. Ca^{2+}) channels (Lester and Jahr, 1990). The specific mGluRs involved in these responses and effects of second messengers linked to these mechanisms on neuronal excitability also remain to be investigated.

SUMMARY

Recent molecular biological information about the structure and pharmacological characteristics of mGluRs subtypes has begun to enhance understanding of cellular mechanisms linked to their activation. It is now apparent that a myriad of pre- and postsynaptic mechanisms exist by which *in situ* expressed mGluRs could modulate cell function in the CNS, and the role of specific mGluR subtypes can now be investigated. More selective agonist and antagonist compounds than those currently known will be needed to sort out the seemingly complex cellular functions of mGluR subtypes. Such agents offer novel approaches to modulate glutamatergic neuronal transmission in very select areas of the CNS.

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Ref. 8 (USSN 10/527,525)

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Markou et al.

Application No.: 10/527,525

Filed: Oct. 14, 2005

For: Methods for treating disorders associated with mGLU receptors including addiction and depression

Art Unit: 1617

Examiner: K. Carter

DECLARATION OF DR. ATHINA MARKOU UNDER 37 CFR § 1.131

Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Athina Markou, states as follows:

1. I am one of the co-inventors of the above-captioned patent application. I am a Professor at the Department of Psychiatry, at the University of California, San Diego and Adjunct Professor at the Molecular and Integrative Neurosciences Department of The Scripps Research Institute. I have published more than 120 journal articles and book chapters, and have received the Efron Award from the American College of Neuropsychopharmacology. I am Section Editor of *Neuropharmacology*, and on the editorial boards of eight other journals, including *Biological Psychiatry*, *Neuropsychopharmacology*, *Behavioral Neuroscience* and *The American Journal on Addictions*. I am also Director of a NIH consortium on the discovery of treatments for depression and nicotine dependence, as well as Principal Investigator on several research grants on nicotine dependence funded by the National Institute on Drug Abuse and the State of California. My curriculum vitae is attached as Exhibit A.

2. I understand that the Examiner has rejected the claims pending in the subject patent application as being obvious. I understand that the Examiner's primary belief underlying the rejection is that both mGluR2/3R antagonists and mGluR5 antagonists have been used to treat drug addiction and substance abuse, and, therefore, it would be obvious to combine a mGluR2/3R antagonist and a mGluR5 antagonist to treat addictive disorders as presently claimed.

3. I understand that the Examiner has cited Fundytus et al., "Attenuation of morphine withdrawal symptoms by subtype-selective metabotropic glutamate receptor antagonists," British J. Pharmacol. 120:1015-20, 1997, and believes that Fundytus et al. reports treatment of withdrawal symptoms with a mGluR2/3 and mGluR5 dual antagonist MCPG.

4. It is my professional opinion that, prior to the subject invention, it would not be obvious to combine a mGluR2/3R antagonist and a mGluR5 antagonist to treat addictive disorders. My opinion is based on the following scientific knowledge and literature reports.

(i) mGlu5 receptors are located postsynaptically. On the other hand, mGlu2/3 receptors are located presynaptically. This is illustrated in the publication by Schoepp, "Unveiling the functions of presynaptic metabotropic glutamate receptors in the central nervous system." J. Pharmacol. Exp. Ther. 299:12-20, 2001 (attached as Exhibit B). The localization of mGluR2/3 and mGluR5 receptors indicates that antagonist actions at the mGluR2/3 increase glutamate transmission, while antagonist actions at the postsynaptic mGluR5 decrease glutamate transmission.

(ii) There are a number of references from the literature suggesting that one should expect opposite neurochemical and behavioral effects of metabotropic glutamate 2/3 receptor (GluR2/3) antagonists and metabotropic glutamate receptor 5 (mGluR5) antagonists. Neurochemically, Mills et al. (J. Neurochem. 79: 835-48, 2001; attached as Exhibit C) examined the role of mGluRs in the increase in extracellular excitatory amino

acids following spinal cord injury, and found that the mGluR5 antagonist MPEP decreased excitatory amino acid concentrations, while treatment with the mGluR2/3 agonist LY 341495 increased excitatory amino acid levels. Xi et al. (J. Pharmacol. Exp. Ther. 300:162-71, 2002; attached as Exhibit D) reported that the mGluR2/3 antagonist LY143495 increased extracellular glutamate in the nucleus accumbens. By contrast, the mGluR5 antagonist MPEP inhibited glutamate release in vitro and in vivo in the corpus striatum (Thomas et al., Neuropharmacology 41: 523-7 2001; attached as Exhibit E) and the periaqueductal grey (de Novellis et al., Eur J Pharmacol 462: 73-81 2003; attached as Exhibit F). Behaviorally, Sharko et al. (Alcohol. Clin. Exp. Res. 32: 67-76, 2008; attached as Exhibit G) studied whether mGluRs modulate the acute sedative-hypnotic properties of ethanol in C57BL/6J mice. The authors found that the mGluR5 antagonist MPEP significantly enhanced both the sedative and hypnotic effects of ethanol; while the mGluR2/3 antagonist LY341495 significantly decreased the sedative hypnotic effects of ethanol.

5. Substance abuse and substance dependence are related but different concepts. Continued substance abuse can often lead to development of substance dependence (or addiction). When subjects with substance dependence cease substance use (i.e., withdrawal), they will usually develop withdrawal symptoms (e.g., depression). It is important to note that substance abuse and substance dependence may usually require different means for treatment and intervention.

6. Fundytus et al. cited by the Examiner does not report that MCPG is effective in treating withdrawal symptoms in rats with morphine dependence. Instead, Fundytus et al. disclosed data which indicate that MCPG prevented the development of morphine dependence in rats which were simultaneously administered with morphine (see, e.g., Fig. 1 of Fundytus et al.).

7. Fundytus et al. reported that treatment with mGluR antagonists, including MCPG, has no effect on withdrawal symptoms in rats that have already developed morphine dependence. This is stated in Fundytus et al., e.g., at page 1018, left column, second and third paragraphs.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: Sept 2, 2008 Athina Markou

Athina Markou, Ph.D.

Attachments: Exhibits A-G.

Journal of Neurochemistry, 2001, **79**, 835–848

Ref. 9 (USSN 10/527,525)

Involvement of metabotropic glutamate receptors in excitatory amino acid and GABA release following spinal cord injury in rat

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Abstract

Spinal cord injury (SCI) leads to an increase in extracellular excitatory amino acid (EAA) concentrations resulting in glutamate receptor-mediated excitotoxic events. The glutamate receptors include ionotropic (iGluRs) and metabotropic (mGluR) receptors. Of the three groups of mGluRs, group-I activation can initiate intracellular pathways that lead to further transmitter release. Groups II and III mGluRs function mainly as autoreceptors to regulate neurotransmitter release. In an effort to examine the role of mGluRs in the increase in EAAs following SCI, we administered AIDA, a potent group-I mGluR antagonist immediately after injury. To determine subtype specific roles of the group-I mGluRs, we evaluated EAA release following LY 367385 (mGluR1 antagonist) and MPEP (mGluR5 antagonist) administration. To evaluate group-II and -III mGluRs we administered APDC (group-II agonist) and L-AP4 (group-III agonist) immediately following injury; additionally, we initiated treatment with CPPG (group-II/-III antagonist) and LY 341495 (group-II antagonist) 5 min prior to injury. Subjects were adult male Sprague–Dawley rats (225–250 g), impact injured at T10 with an NYU impactor (12.5 mm drop). Agents were injected into the epicenter of injury, amino acids where collected by microdialysis fibers inserted 0.5 mm caudal from the edge of the impact region and quantified by HPLC. Treatment with AIDA significantly decreased extracellular EAA and GABA concentrations. MPEP reduced EAA concentrations without affecting GABA. Combining LY 367385 and MPEP resulted in a decrease in EAA and GABA concentrations greater than either agent alone. L-AP4 decreased EAA levels, while treatment with LY 341495 increased EAA levels. These results suggest that mGluRs play an important role in EAA toxicity following SCI.

Keywords: excitotoxicity, metabotropic glutamate receptors, spinal cord injury.

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The initial damage caused by injury to the CNS is often limited in area (McIntosh *et al.* 1998). However, there is a progressive expansion of the damaged area over time due to secondary effects. A primary cause of secondary damage is excitatory amino acid (EAA) toxicity. Following injury (e.g. ischemia in the brain or mechanical injury to the spinal cord), brief, but large, amounts of glutamate and aspartate are released at the injury site (Panter *et al.* 1990; Liu *et al.* 1991; McAdoo *et al.* 1999). This rise in extracellular EAAs may be due to exocytosis, inhibited or reversed uptake, blood brain barrier breakdown, and cell lysis (McAdoo *et al.* 2000; Rothstein *et al.* 1996; Tanaka *et al.* 1997; Vera-Portocarrero *et al.* 1999). The high levels of extracellular EAAs set up an excitotoxic cycle in which extracellular glutamate activates glutamate receptors, leading to more glutamate release that activates more glutamate receptors. The glutamate receptors are divided into two major types: (i) ionotropic receptors (iGluR): NMDA, AMPA and kainate receptors, and (ii) metabotropic receptors: metabotropic glutamate receptors (mGluR), groups I, II and III. Since the mGluRs are G-protein coupled, their activation can initiate

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Abbreviations used: ACSF, artificial cerebral spinal fluid; AIDA, 1-aminoindan-1,5-dicarboxylic acid; APDC, (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate; L-AP4, L-2-amino-4-phosphonobutyric acid; BLA, basolateral amygdala; CPPG, (*R*,*S*)-α-cyclopropyl-4-phosphonophenylglycine; DAG, diacyglycerol; EAA, excitatory amino acid; iGluR, ionotropic glutamate receptor; LY, 341495 (α*S*)-α-amino-α-[(1*S*,2*S*)-2-carboxycyclopropyl]-9*H*-xanthine-9-propanoic acid; LY 367385, 2-methyl-4-carboxyphenylglycine; MK-801, (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohepten-5,10-imine; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; mGluR, metabotropic glutamate receptor; PKC, protein kinase C; SCI, spinal cord injury.

numerous intracellular signaling pathways that have a variety of complex and long-lasting effects.

Metabotropic glutamate receptors are divided into three groups based on sequence homology, transduction mechanisms and pharmacological profiles: (i) group I consists of mGluR1 and mGluR5; (ii) group II consists of mGluR2 and mGluR3; and (iii) group III consists of mGluR4, mGluR6, mGluR7 and mGluR8. Splice variants have been found for mGluR1 (α,β,c,e) mGluR4 (a,b) and mGluR5 (a,b) (Conn and Pin 1997). All subtypes of mGluRs, except for mGluR6 and mGluR8, are found in the spinal cord (Duvoisin *et al.* 1995; Valerio *et al.* 1997; Berthele *et al.* 1999). Group-I mGluRs are coupled to phosphatidylinositol hydrolysis, which leads to increases in intracellular Ca^{2+} levels and activation of protein kinase C (PKC) via diacyglycerol (DAG) formation. Increases in free cytosolic Ca^{2+} and activation of PKC are components of pathways that lead to cell death (Choi 1992). Activation of presynaptic group-I mGluRs enhances the release of glutamate (Herrero *et al.* 1992), which is facilitated by a PKC-mediated inhibition of presynaptic K^+ channels (Pin and Duvoisin 1995). Evidence for group-I receptors' involvement in neurodegeneration comes from injections of (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (agonist at group-I mGluRs), which has neurotoxic effects in the rat hippocampus and caudate nucleus (McDonald and Schoepp 1992; Sacaan and Schoepp 1992; McDonald *et al.* 1993) and in models of global ischemia (Henrich-Noack and Reymann 1999; Pellegrini-Giampietro *et al.* 1999). The neurotoxic effects are blocked by (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohepten-5,10-imine (MK-801), a noncompetitive, open channel NMDA antagonist, suggesting NMDA receptor involvement. There appears to be a synergistic effect between mGluRs and iGluRs, where activation of mGluRs potentiates iGluR responses, which in turn potentiate mGluR activation (Gereau and Heinemann 1998; Alagarsamy *et al.* 1999). Additional evidence for involvement of group-I mGluRs in neurotoxicity is shown by a selective mGluR1 antagonist, 2-methyl-4-carboxyphenylglycine (LY 367385) having neuroprotective effects using *in vitro* and *in vivo* models of excitotoxic death (Bruno *et al.* 1999; Mills *et al.* 2001b). Antagonists to group-I mGluRs have been shown to improve recovery of compound action potentials following spinal cord injury (Agrawal *et al.* 1998). Thus, activation of group-I mGluRs may contribute to excitotoxicity that leads to cell death.

In contrast, activation of group-II and III mGluRs may attenuate neuronal death (Bruno *et al.* 1994, 1995; Buisson and Choi 1995; Orlando *et al.* 1995; Turetsky *et al.* 1995; Buisson *et al.* 1996). Bruno *et al.* (1994) have shown that the group-II agonist (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine inhibits kainate and NMDA-induced neurotoxicity in cortical neurons. Furthermore, activation of group-II mGluRs attenuates neuronal injury *in vitro*, improves behavioral recovery following traumatic brain injury (Allen *et al.* 1999), and is protective in global ischemia (Bond *et al.* 1999). Group-III mGluR activation attenuates post-traumatic neuronal death *in vitro* (Faden *et al.* 1997). Activation of presynaptic group-II and -III mGluRs depresses synaptic transmission in basolateral amygdala (BLA) neurons (Rainnie and Shinnick-Gallagher 1992), which is enhanced 30-fold in kindled BLA neurons (Neugebauer *et al.* 1997). The inhibition of neurotransmitter release by activation of presynaptic group-II and -III mGluRs may occur via G-protein-mediated inhibition of high voltage-activated Ca^{2+} channels (Trombley and Westbrook 1992; Sahara and Westbrook 1993; Chavis *et al.* 1994; Ikeda *et al.* 1995; Choi and Lovinger 1996) and may involve glial interactions (Bruno *et al.* 1997). Thus, activation of group-II and -III mGluRs may attenuate neuronal death following injury by inhibiting the excitotoxic cascade.

Recently it was demonstrated that a mGluR group-I selective antagonist, 1-aminoindan-1,5-dicarboxylic acid (AIDA), decreases extracellular glutamate concentrations and is neuroprotective in gray and white matter following spinal cord injury (SCI; Mills *et al.* 2000, 2001b). Here, we extend these studies to examine different dosing regiments of AIDA and to determine which group-I subtype, mGluR1 or mGluR5, mediates the increase in EAA release using the mGluR1 selective antagonist, LY 367385, and the mGluR5 selective antagonist, 2-methyl-6-(phenylethynyl)-pyridine (MPEP). Additionally, we examined the roles of group-II and -III mGluRs in EAA and GABA release following SCI by administering agonists for group-II (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate (APCD), and group III, L-2-amino-4-phosphonobutyric acid (L-AP4) immediately following SCI. To further evaluate group-II and -III mGluRs, we initiated treatment preinjury with the antagonists (*R*,*S*)-α-cyclopropyl-4-phosphonophenylglycine (CPPG; group-II/-III) and (α*S*)-α-amino-α-[(1*S*,2*S*)-2-carboxycyclopropyl]-9*H*-xanthine-9-propanoic acid (LY 341495; group II). Table 1 provides a summary of the compounds used and their actions.

Table 1 Summary of mGluRs and pharmacological agents

Group	Subtypes	Transduction mechanisms	Compounds	Actions
I	1	+ PLC	AIDA, LY 367385	Antagonists
	5	+ PLC	AIDA, MPEP	Antagonists
II	2,3	− AC	APDC	Agonist
			CPPG, LY 341495	Antagonist
III	4,6,7,8	− AC	L-AP4	Agonist
			CPPG	Antagonist

Materials and methods

Experimental animals

Subjects were male Sprague–Dawley rats, 225–250 g, obtained from Harlan Sprague–Dawley, Inc. and housed with a light/dark cycle of 12 h/12 h. Experimental procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Sixty-three rats were divided into 16 groups ($n = 3$–5 each): injury only; injury + agent: vehicle (post injury: 2 μL); vehicle (post injury: 4 μL); vehicle (pre through post injury: 4 μL), AIDA (10 nmol, 200 nmol), LY 367385 (200 nmol), MPEP (200 nmol), LY 367385 + MPEP (200 nmol each), APDC (500 nmol), L-AP4 (500 nmol, 1.0 μmol), CPPG (10 nmol, 50 nmol), and LY 341495 (30 nmol, 100 nmol).

Injury production

Spinal cord contusion injury was produced as previously described (Gruner 1992; Huang and Young 1994; Hulsebosch *et al.* 2000). Briefly, subjects were anesthetized by an intraperitoneal injection of pentobarbital (40 mg/kg). Anesthesia was considered complete when there was no flexor withdrawal in response to noxious foot pinch. The subjects' backs were shaven, an incision made to expose the vertebral column, and laminectomies were performed to expose spinal segments T10–T11. Spinal cord injury was produced using the New York University (NYU) injury device. A 10-g weight, 2.0 mm in diameter, was dropped from 12.5 mm onto the exposed cord.

Microdialysis sampling

Microdialysis sampling was conducted as previously described (Liu and McAdoo 1993). Briefly, microdialysis fibers were prepared by coating dialysis fibers (Spectrum Laboratories, Rancho Dominguez, CA, USA) with a thin layer of silicone rubber except for a 2-mm dialysis zone. Fused silica tubes (Polymicro Technologies, Phoenix, AZ, USA) were glued into the ends of the microdialysis tubing just outside the spinal cord to provide inlet and outlet connections. Including their coating, individual fibers had an external diameter of 180 μm. The microdialysis fiber was inserted laterally through the cord 0.5 mm from the caudal edge of the impact zone (1.5 mm from the epicenter of injury) with one end attached to a syringe pump containing artificial cerebrospinal fluid (ACSF; containing, in mM: 151.1 Na^{2+}, 2.6 K^{+}, 0.9 Mg^{2+}, 1.3 Ca^{2+}, 122.7 Cl^{-}, 21.0 HCO_3^{-} and HPO_4^{2-}). The ACSF was bubbled with 95% O_2/5% CO_2 prior to each experiment to adjust the pH to 7.2. Samples were collected at the outlet end of the tubing in small plastic vials on ice. ACSF was pumped through the fiber for 1 h to allow insertion damage to subside; then three sequential 15 min samples were collected for baseline values. The cord was then injured and four sequential 15 min samples were collected followed by six sequential 30 min samples, yielding a total collection time of 4 h after injury. Following injury, the cord was covered with mineral oil maintained at 37–38°C. Body temperature was maintained at 37–38°C using a homothermic blanket system (Harvard Apparatus, Holliston, MA, USA). Naphthalene dicarboxaldehyde derivatives of amino acids were analyzed by HPLC using fluorescence detection as previously (Liu and McAdoo 1993).

Drug and vehicle administration

All compounds were obtained from Tocris (Ballwin, MO, USA), prepared in ACSF, and injected stereotaxically into the epicenter of impact through a 30-gauge needle coupled to a syringe pump (CMA/Microdialysis AB, Stockholm, Sweden), at a depth of 1 mm immediately following SCI (within 3 min). Two different dosing regiments of AIDA (pH 7.4) were used: (i) A total volume of 2 μL of a 5.0-mM solution (10 nmol total) was injected during the first 5 min after injury. (ii) A total volume of 4 μL of a 50-mM solution (200 nmol total) was injected during the first 20 min following injury. LY 367385 (pH 7.4) and MPEP (pH 7.3) were both administered by injecting 4 μL of a 50-mM solution (200 nmol total). LY 367385 + MPEP (200 nmol each; pH 7.3) was administered by injecting 4 μL of a solution that was 50 mM for each. APDC and L-AP4 (pH 7.4) were administered by injecting 4 μL of a 125-mM solution (500 nmol total). Additionally, another group of animals received 4 μL of a 250-mM solution of L-AP4 (1.0 μmol total). LY 367385, MPEP, LY 367385 + MPEP, APDC, and L-AP4 were administered during the first 20 min following injury. CPPG (30 nmol or 50 nmol) and LY 341495 (30 nmol or 100 nmol) were injected in 4 μL of ACSF over a 20-min interval starting 5 min prior to injury. The doses of AIDA selected were based on previous work demonstrating the affect of AIDA on glutamate release and its neuroprotective actions following SCI (Mills *et al.* 2000, 2001b). For LY 367385, MPEP, APDC, and L-AP4, we selected the lowest doses that gave the maximum behavioral and neuroprotective effects (Bruno *et al.* 1999, 2000; Chapman *et al.* 1999, 2000; Mills *et al.* 2001b,c). CPPG and LY 341495 doses were based on previous pharmacological studies (Jane *et al.* 1996; Kingston *et al.* 1998; Johnson *et al.* 1999; Naples and Hampson 2001).

One concern with dose selection, particularly with the group-I antagonists, is the interactions of these compounds with iGluRs. For example, MPEP has been reported to inhibit NMDA receptor activity at concentrations above 20 μM in cultured rat cortical neuronal cells (Movsesyan *et al.* 2001), while other studies report no effects on NMDA receptors at 100 μM in *Xenopus laevis* oocytes (Gasparini *et al.* 1999). LY 367385 affects iGluRs at IC_{50} values > 10 mM (Bruno *et al.* 1999); however, one study reports a slight enhancement of NMDA and AMPA responses after treatment with 50 mM of LY 367385 in the ventrobasal thalamus (Salt and Binns 2000). To calculate the maximum possible concentration of an agent at the microdialysis fiber, we assumed that the spinal cord could be approximated by a cylinder (radius of 1.5 mm and length 3.0 mm; injections were performed 1.5 mm from the dialysis fiber and assuming equal diffusion in both directions) and calculated its volume. The concentration of the agent was calculated assuming uniform diffusion throughout the cylinder. To illustrate, injection of 200 nmol of LY367385 would give a maximum concentration at the micordialysis fiber of 9.4 mM, which is below the concentrations reported to effect iGluRs (Bruno *et al.* 1999). It is important to note that actual concentrations of the agents at the microdialysis fiber are probably much less due to uptake and tortuosity (a measure of how diffusing molecules are hindered by cellular obstructions; Lieberman *et al.* 1995; Lonser *et al.* 1998; Nicholson 1999).

Statistical analysis

Glutamate, aspartate, and GABA concentrations were analyzed using a two-sample *t*-test with an α level set *a priori* at 0.05 at individual time points to test for significant differences between injury only, injury + vehicle, and injury + agent treatment groups.

When indicated, *post hoc* pairwise comparisons between treatment groups were made using Fisher's least significant difference (LSD) test. Data are expressed as means ± SEM.

Results

Extracellular EAA and GABA concentrations following SCI

Following SCI there is an increase in extracellular glutamate and aspartate concentrations that reach maximum levels of 49.2 ± 8.0 μM and 14.6 ± 2.1 μM, respectively, which slowly return to baseline levels by 120 min after injury (Figs 1a and b). Compared with injury alone, treatment with each vehicle (ACSF) regiment did not significantly affect extracellular glutamate or aspartate levels (Figs 1a and b). Normal basal levels of GABA in the spinal cord are extremely low (below 0.03 μM; data not shown); however, following injury extracellular GABA concentrations rise to reach maximum levels of 4.8 ± 0.8 μM and remain elevated through 30 min following injury (1.1 ± 0.4 μM; Fig. 1c). By 45 min following injury extracellular GABA levels return to baseline levels. Therefore, GABA concentrations are reported for only the first 30 min following injury. Compared with injury alone, vehicle treatment (any volume or duration) did not affect extracellular GABA levels at any time point analyzed (Fig. 1c).

AIDA effects on extracellular EAA concentrations

Administration of 10 nmol of AIDA, a mGluR group-I selective antagonist, during the first 5 min of injury significantly reduced extracellular glutamate concentrations to 27.7 ± 2.5 μM and aspartate concentrations to 4.7 ± 1.1 μM (Figs 2a and c; $p < 0.05$). Increasing the dose of AIDA to 200 nmol, given over a 20-min period following SCI, did not result in a further decrease in extracellular glutamate or aspartate concentrations (Figs 2b and d). Maximum glutamate and aspartate concentrations following 200 nmol of AIDA were 32.9 ± 6.0 μM and 6.6 ± 1.8 μM, respectively. Since both 10 and 200 nmol of AIDA produced similar effects, it is likely that saturation of group-I mGluRs occurs below 10 nmol of AIDA.

LY 367385 and MPEP effects on extracellular EAA concentrations

Administration of the mGluR1 specific antagonist LY 367385 (200 nmol over 20 min) did not affect the initial increase in extracellular glutamate or aspartate concentrations following SCI (Figs 3a and d). Extracellular glutamate and aspartate levels at 15 min following SCI were 44.9 ± 4.9 μM and 11.8 ± 1.6 μM in the LY 367385-treated group. Administration of the mGluR5 specific antagonist MPEP (200 nmol over 20 min) significantly reduced both glutamate and aspartate levels immediately following SCI (Figs 3b and e; $p < 0.05$). Extracellular

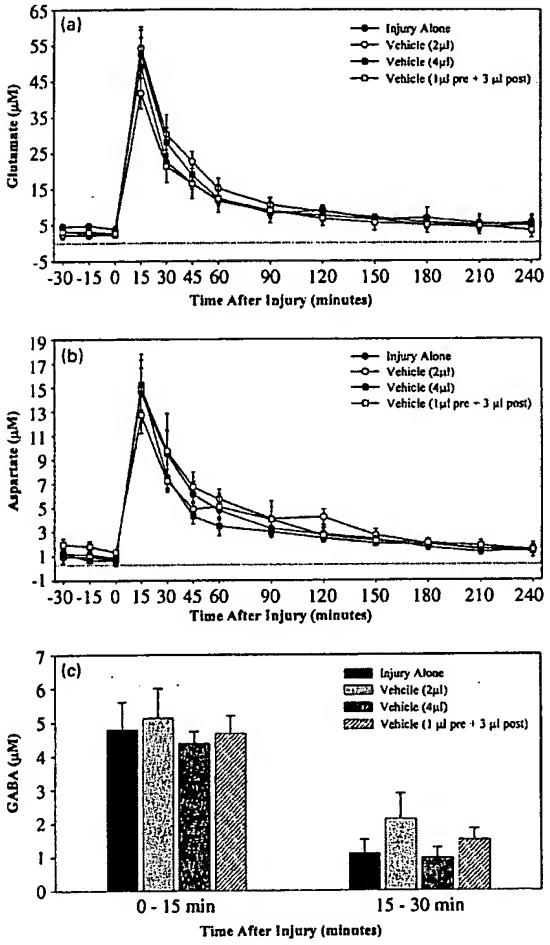

Fig. 1 Time course of extracellular glutamate (a), aspartate (b), and GABA (c) concentrations after SCI for injured only animals and each vehicle treatment. Vehicle treatments did not significantly affect extracellular glutamate, aspartate, or GABA concentrations after SCI. Data are means ± SEM.

glutamate and aspartate levels at 15 min following SCI were 37.2 ± 4.7 μM and 8.9 ± 1.0 μM in the MPEP-treated group. When LY 367385 and MPEP were administered together, extracellular glutamate and aspartate concentrations were reduced further than when each agent was administered separately to 19.5 ± 5.4 μM and 4.6 ± 1.0 μM, respectively (Figs 3c and f).

Group-I antagonists' effects on extracellular GABA concentrations

Following SCI, treatment with AIDA (10 and 200 nmol) significantly reduced extracellular GABA levels compared with injury alone and the vehicle-treated group within the first 15 min of injury (Fig. 4a; $p < 0.05$). Treatment with

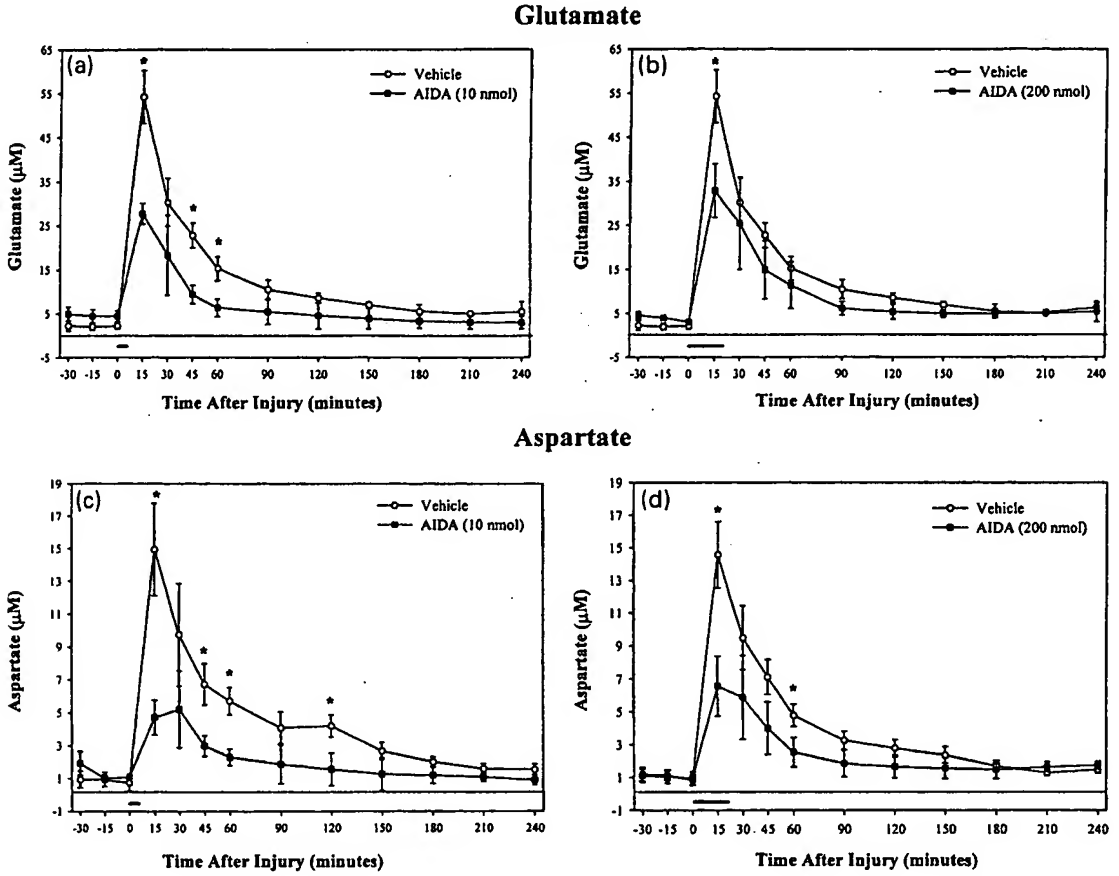

Fig. 2 Time course of extracellular glutamate (a and b) and aspartate (c and d) concentrations after SCI with 10 nmol (a and c) or 200 nmol (b and d) of AIDA. Following SCI, there is an increase in extracellular glutamate and aspartate concentrations that slowly returns to baseline values by 120 min after injury. Treatment with AIDA at 10 nmol (a) or 200 nmol (b) significantly reduced extracellular glutamate levels compared with the vehicle-treated group during the first 15 min following SCI. Similarly, aspartate levels were decreased in the AIDA-treated group [10 nmol (c) and 200 nmol (d)] during the first 15 min after injury compared with the vehicle-treated group. Data are means ± SEM. *Indicates a statistically significant difference between AIDA-treated and vehicle-treated ($p < 0.05$). Treatment duration is indicated by a horizontal line above the *x*-axis.

LY 367385 or MPEP did not affect extracellular GABA concentrations after SCI. However, combining LY 367385 and MPEP resulted in a significant decrease in extracellular GABA concentrations compared with injury alone and the vehicle-treated group within the first 15 min of SCI (Fig. 4a; $p < 0.05$). By 30 min after SCI, there was not a statistically significant difference between injury alone or the vehicle-treated group versus any group-I agent treated group (Fig. 4b).

APDC and L-AP4 effects on extracellular EAA and GABA concentrations

Following SCI, 500 nmol of APDC did not affect the initial rise in extracellular glutamate or aspartate concentrations (Figs 5a and c). Glutamate and aspartate levels at 15 min following SCI were 37.8 ± 7.3 μM and 12.7 ± 2.4 μM, respectively, in the APDC-treated group. Administration of L-AP4 (500 nmol) significantly reduced extracellular glutamate levels up to 30 min following SCI. Glutamate and aspartate levels at 15 min following SCI were 30.9 ± 6.2 μM and 9.0 ± 1.7 μM, respectively, in the L-AP4 (500 nmol)-treated group (Figs 5b and d; $p < 0.05$). Since EC_{50} values for L-AP4 vary greatly between mGluR4 and mGluR7 subtypes (Conn and Pin 1997), we tested a higher dose of L-AP4 (1.0 μmol). Only a slight decrease, which was not statistically significant compared with 500 nmol of L-AP4, in extracellular glutamate and aspartate concentrations was seen (Figs 5b and d). Treatment with

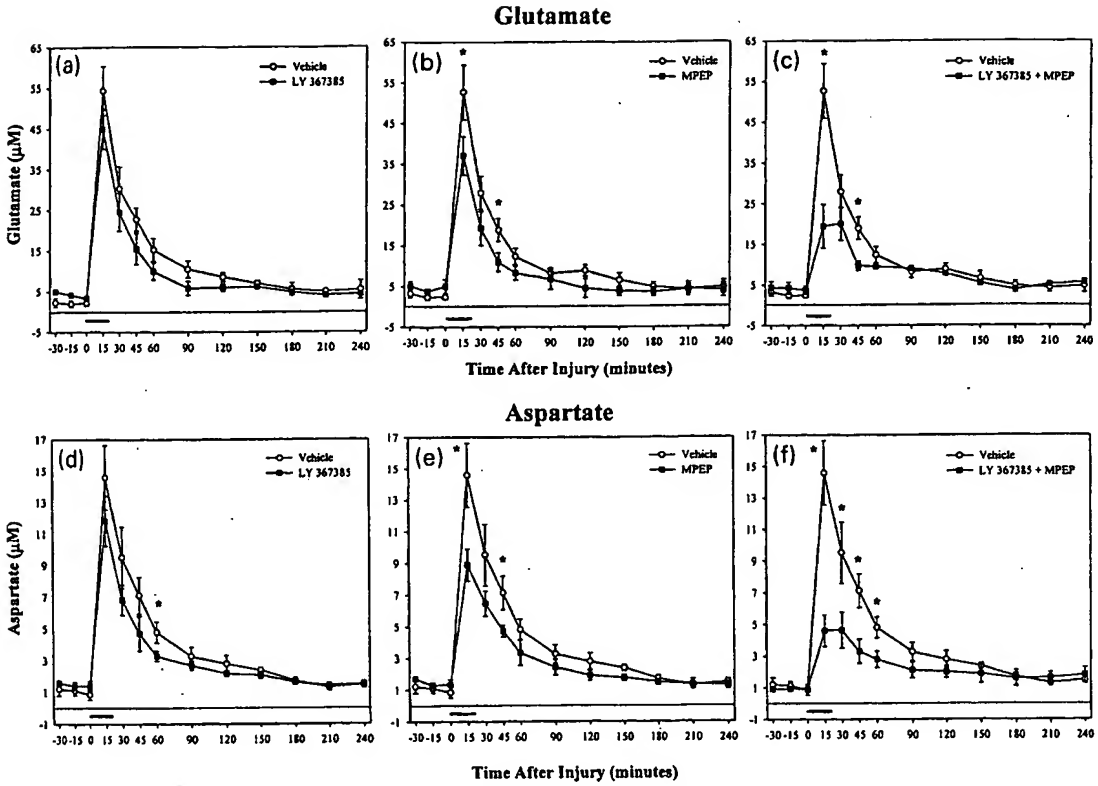

Fig. 3 Time course of extracellular glutamate (a–c) and aspartate (d–f) concentrations after SCI in LY 367385- (a and d), MPEP- (b and e), and LY 367385 + MPEP-treated (c and f) groups. MPEP (b), but not LY 367385 (a), reduced extracellular glutamate concentrations following SCI. However, combining LY 367385 and MPEP (c) resulted in a greater decrease in extracellular glutamate concentrations than either agent alone. Similar results were obtained for aspartate levels following SCI for LY 367385 (d), MPEP (e), and LY 367385 + MPEP (f). Data are means ± SEM. *Indicates a statistically significant difference between treatment groups ($p < 0.05$). Treatment duration is indicated by a horizontal line above the *x*-axis.

APDC and L-AP4 did not significantly affect extracellular GABA concentrations at any time point (Fig. 6a).

CPPG and LY 341495 effects on extracellular EAA and GABA concentrations

Treatment with CPPG (10 or 50 nmol) from 5 min prior through 15 min post injury did not have a significant effect on extracellular glutamate or aspartate levels (Figs 7a and c). However treatment with LY 341495 (30 and 100 nmol) from 5 min prior through 15 min after injury increased extracellular glutamate and aspartate levels 15–30 min post injury compared with vehicle control ($p < 0.05$; Figs 7b and d). Neither CPPG nor LY 341495 treatment affected extracellular GABA concentration following injury (Fig. 6b).

Discussion

The present study demonstrates that individual subtypes of mGluRs differentially influence extracellular EAA and GABA concentrations following SCI. Within the group-I mGluRs, antagonist treatment to mGluR1 had less of an effect on extracellular EAA concentrations than did antagonist treatment against mGluR5. However, combining treatments had a greater effect than either treatment alone. Activation of group-III, but not group-II, mGluRs following SCI reduced extracellular EAA levels without affecting GABA concentrations. Antagonist treatments to group-II mGluRs initiated prior to injury increased EAA concentrations above injury-induced values. These results suggest that mGluRs play important roles in the initial events that lead to excitotoxicity following CNS injury.

Group-I mGluRs and EAA release

Activation of group-I mGluRs enhances neurotransmitter release (Herrero *et al.* 1992), which is facilitated by a PKC-mediated inhibition of presynaptic K^+ channels (Pin and Duvoisin 1995). The activation of group-I mGluRs may increase EAA release by modulating the membrane

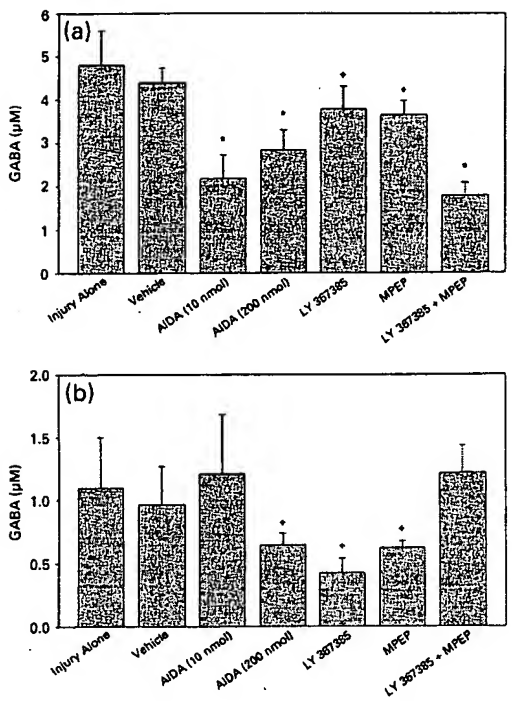

Fig. 4 Extracellular GABA concentrations 0–15 min (a) and 15–30 min (b) following SCI. Treatment with AIDA (10 and 200 nmol) and LY 367385 + MPEP reduced extracellular GABA concentrations immediately following SCI compared with the vehicle-treated group (a). However, 15–30 min after SCI, no treatment group was significantly different from the vehicle-treated group (b). Data are means ± SEM. *Indicates a statistically significant difference compared with vehicle-treated; + indicates a statistically significant difference compared with LY 367385 + MPEP ($p < 0.05$).

potential through inhibition of IK_{leak} and IK_M or increase the firing rate by inhibiting IK_{AHP} (Pin and Duvoisin 1995). A previous report showed that the group-I antagonist AIDA reduced extracellular glutamate concentrations following SCI (Mills *et al.* 2000). The present study extends these results to demonstrate that higher doses of AIDA do not further reduce EAA levels following SCI. Since AIDA antagonizes both mGluR1 and mGluR5 (Schoepp *et al.* 1999), its effects do not reveal which group-I subtype, mGluR1, mGluR5, or both, mediates these responses.

To test involvement of individual group-I subtypes in EAA release following SCI, we used the mGluR1 specific antagonist LY 367385 and the mGluR5 specific antagonist MPEP. MPEP, but not LY 367385, significantly decreased glutamate and aspartate extracellular concentrations immediately following SCI. This suggests that mGluR5 plays a greater role than mGluR1 in modulating the increase in extracellular glutamate and aspartate concentrations after SCI. Group-I mGluRs can potentiate iGluR responses and iGluRs can facilitate mGluR activation (Aniksztejin *et al.* 1992; Bleakman *et al.* 1992; Kelso *et al.* 1992; Glaum and Miller 1993; Ugolini *et al.* 1997, 1999; Budai and Larson 1998; Alagarsamy *et al.* 1999; Salt and Binns 2000). Inhibition of this synergistic relationship is one mechanism by which antagonism of group-I mGluRs may affect neurotransmitter release. Since MPEP, but not LY 367385, inhibited glutamate release, it may be that mGluR5 is more tightly coupled than mGluR1 to iGluR potentiation in the spinal cord.

Group-I mGluRs have been shown to inhibit or increase transmitter release depending on neuronal activity (Herrero *et al.* 1998; Rodriguez-Moreno *et al.* 1998; Sistiaga and Sanchez-Prieto 2000). A functional switch that is mediated by receptor phosphorylation has been proposed to account for these conflicting actions (Herrero *et al.* 1998). Under basal conditions, activation of group-I mGluRs facilitates release, whereas activation during times of excess extracellular glutamate inhibits release (Herrero *et al.* 1998). If mGluR1 undergoes the switch from facilitation to inhibition while mGluR5 remains in a facilitatory state, mGluR1 antagonists would not have as great of an effect as mGluR5 antagonists on transmitter release, consistent with observations in the current study. Expression levels of mGluR5 are much higher than those of mGluR1 in the rat thoracic spinal cord (Mills *et al.* 2001a). Thus, a simpler explanation is that antagonism of mGluR5 has a greater effect simply because there is more mGluR5 than mGluR1 in the rat thoracic spinal cord.

However, when antagonists for each subtype were combined, a greater decrease in extracellular EAAs was seen than when agents were tested individually. The different subcellular localizations of mGluR1 and mGluR5 in the spinal cord (Alvarez *et al.* 2000) may explain why a combination of mGluR1 and mGluR5 antagonists is more effective than the individual treatments. mGluR5 appears to be targeted to distal dendritic regions (except in lamina II), whereas mGluR1 is seen throughout the somatodendritic membrane (Alvarez *et al.* 2000). Furthermore, mGluR5 is more highly expressed than mGluR1 in the rat thoracic spinal cord (Mills *et al.* 2001a). Thus, there may be more mGluR5 in synaptic zones, while the less expressed mGluR1 is more dispersed. Therefore, antagonists against mGluR5 would be expected to have a greater effect than mGluR1 antagonists, and mGluR1 treatments might not produce an observable effect on EAA release. Combining the two treatments would affect both the large population of mGluR5s in the dendritic zones (directly affecting EAA release) and the mGluR1s in the cell soma (affecting membrane potential), which would produce a larger effect than either treatment alone. To our knowledge there is currently no study that specifically addresses mGluR1 effects on mGluR5 responses or mGluR5 effects on mGluR1 responses that would help assess this possibility.

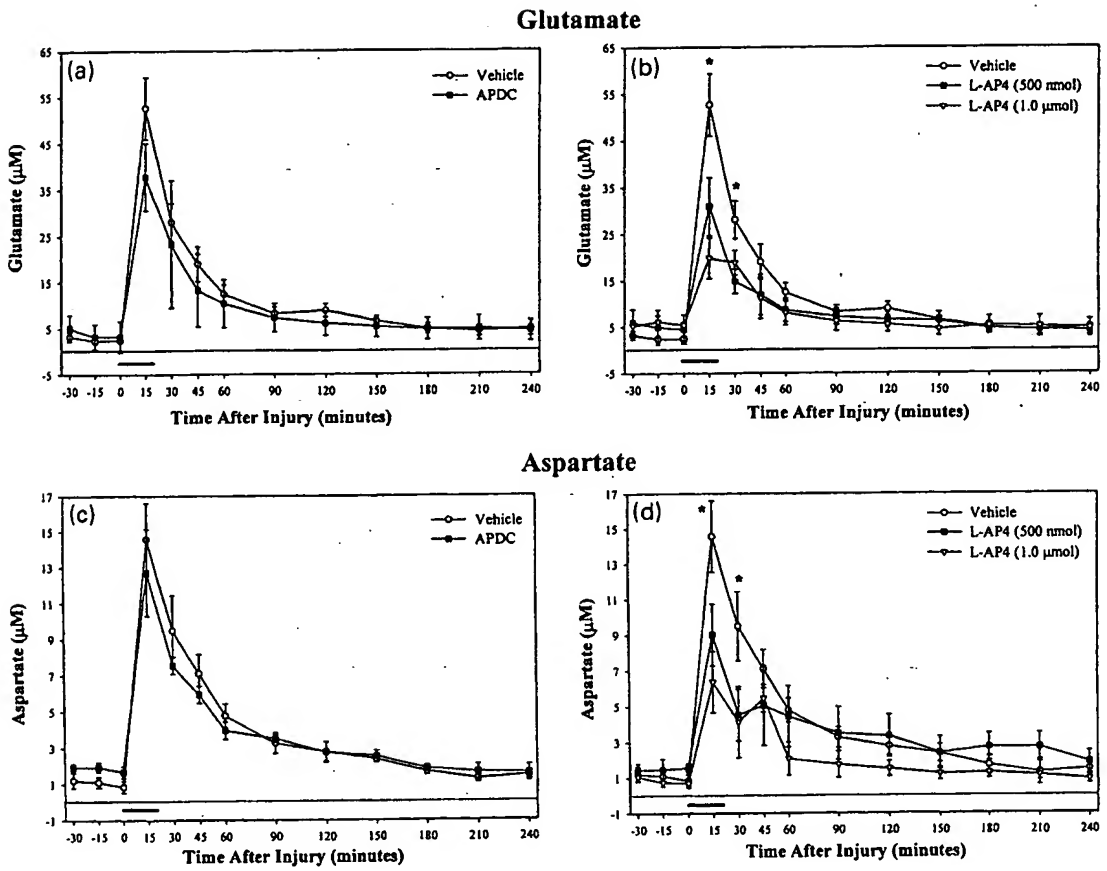

Fig. 5 Time course of extracellular glutamate (a and b) and aspartate (c and d) concentrations after SCI in APDC- (a and c) or L-AP4-treated (b and d) groups. L-AP4, but not APDC treatment decreased extracellular glutamate and aspartate concentrations following SCI. Data are means ± SEM. *Indicates a statistically significant difference between the vehicle and treatment groups ($p < 0.05$). Treatment duration is indicated by a horizontal line above the *x*-axis.

Group-II and III mGluRs and EAA release

The role of group-II and -III mGluRs as autoreceptors to regulate neurotransmission in the CNS is well established. The primary mechanism by which group-II and -III mGluRs regulate neurotransmitter release is presumably through inhibition of Ca^{2+} channels (Pin and Duvoisin 1995). However, other mechanisms of modulating neurotransmission independent of Ca^{2+} channel modulation have been suggested. For example, mGluR autoreceptors may affect presynaptic release machinery (Bushell *et al.* 1999; Gerber *et al.* 2000). In autaptic hippocampal cultures, mGluR7 is colocalized with synaptophysin, suggesting a modulation of neurotransmitter release that is 'downstream' of Ca^{2+} entry (Bushell *et al.* 1999).

Allen *et al.* (1999) demonstrated that the group-II agonist LY 354740 attenuates injury-induced glutamate release in neuronal-glial cultures at 2 h post injury. However, in the current study the group-II agonist APDC did not reduce extracellular EAA concentrations following SCI. In the spinal cord, group-II and -III mGluRs are found presynaptically (Ohishi *et al.* 1995; Azkue *et al.* 2000, 2001). Group-II mGluRs appear to be localized in the preterminal area removed from transmitter release sites (Azkue *et al.* 2000; Shigemoto *et al.* 1997), whereas mGluR4a (group-III mGluR) is clustered along the presynaptic specialization (Azuke *et al.* 2001). The lack of effect of APDC on extracellular EAA levels may be due to the cellular localization of group-II mGluRs in the adult rat spinal cord (farther from the synapse than group III). Excitatory, inhibitory, dual, and mixed effects of group-II mGluRs have been found in the spinal cord (Bond and Lodge 1995; Cao *et al.* 1995, 1997; King and Liu 1996; Bond *et al.* 1997; Stanfa and Dickenson 1998; Dong and Feldman 1999). Thus, another possibility is that activation of spinal group-II

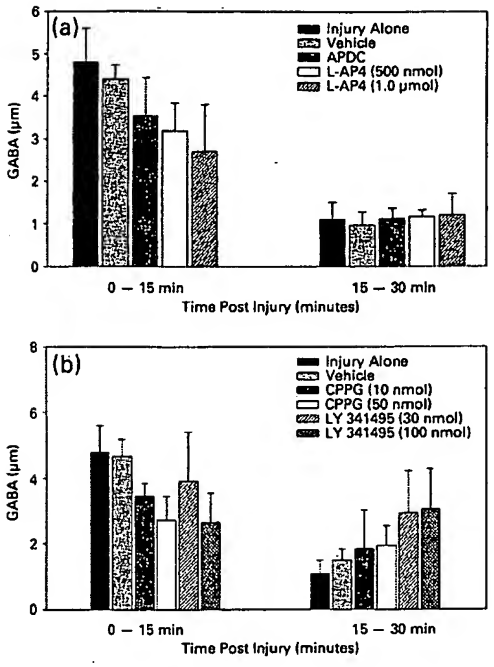

Fig. 6 Extracellular GABA levels after postinjury treatment (0–20 min) following administration of vehicle, APDC, or L-AP4 (a) and treatment initiated pre-injury (– 5–15 min) with vehicle, CPPG, or LY 341495 (b). Post-injury treatment with agonists to group II (APDC) or group III (L-AP4) did not affect extracellular GABA concentrations during the first 30 min of injury. Treatment initiated pre-injury with antagonists to group II (CPPG) or group III (LY 341495) did not affect extracellular GABA concentrations during the first 30 min after injury. Data are means ± SEM.

mGluRs has both inhibitory and excitatory effects, producing an end result of no overall effect (Neugebauer *et al.* 2000).

Interestingly, treatment 5 min prior through 15 min post-SCI with the group-II antagonist LY 341495 potentiated the release of glutamate. This potentiation of EAA release was not seen using the group-II and -III antagonist CPPG. CPPG is more selective for group-III mGluRs (Jane *et al.* 1996); therefore, it appears that antagonism of group-II but not group-III mGluRs potentiates the release of EAAs. Since mGluR3 may be more highly expressed in the spinal cord than mGluR2 (Ohishi *et al.* 1993, 1998; Berthele *et al.* 1999) and LY 341495 is more potent at mGluR3 (Kingston *et al.* 1998), it is likely that inhibition of mGluR3 mediates the potentiation of EAA release after treatment with LY 341495. In normal animals, group-II antagonists reduce thresholds to mechanical stimuli, suggesting that there may be tonic group-II activity in the spinal cord (Dolan and Nolan 2000; Neugebauer *et al.* 2000). Inhibiting this tonic activity prior to SCI may inhibit intracellular signaling pathways that would normally reduce EAA release. However, it is unclear why antagonism of group-III mGluRs did not affect extracellular EAA levels when treatment with the group-III agonist L-AP4 did reduce EAA levels. Since mGluR-mediated responses are tightly regulated by receptor phosphorylation (Peavy and Conn 1998; Saugstad *et al.* 1998; Macek *et al.* 1999; Schaffhauser *et al.* 2000), it is possible that the activation states of group-II and group-III mGluRs change such that group-III mGluRs play a greater role in modulating neurotransmitter release after injury.

GABA release following SCI

The current study is in agreement with a previous report of an increase in extracellular GABA concentrations immediately following contusion injury to the spinal cord (Liu and McAdoo 1993). The temporal increase in extracellular GABA concentrations parallels the rise in extracellular EAAs; both are maximally increased within the first 15 min following SCI. Most of the increase in extracellular GABA after SCI is probably due to the destruction of GABAergic neurons by the initial mechanical damage and release of GABA from un-injured neurons due to the excitation induced from the large increase in extracellular EAAs. If similar mechanisms are responsible for the increase in extracellular GABA levels and EAAs following SCI, then it would be expected that treatments with mGluR agents that affect extracellular EAAs would also affect GABA levels. This is in fact what is seen in the current study: agents that decreased extracellular EAA levels (AIDA and LY 367385 + MPEP) produced the greatest decreases in extracellular GABA concentrations. Additional support comes from the results of treatment with LY 341495 and CPPG. Treatment with LY 341495 resulted in an increase in EAA levels at 30 min following SCI. This rise in EAA was accompanied by an increase in GABA levels, whereas treatment with CPPG did not affect extracellular EAA or GABA levels. Presynaptic mGluR2/3s are associated with GABA terminals in other regions of the CNS and may modulate GABA release from synaptic terminals (Hayashi *et al.* 1993; Ohishi *et al.* 1993, 1994; Stefani *et al.* 1994; Neki *et al.* 1996; Petralia *et al.* 1996). In the spinal cord, mGluR2/3 colocalizes with GABA, although there appears to be segregation between mGluRs and GABA immunopositive perikarya (Jia *et al.* 1999). This modest level of colocalization may explain the relative lack of effect on extracellular GABA levels using the group-II and -III agents in the current study.

Relationship between effects on EAA release and neuroprotection

Recently it was reported that treatment with LY 367385 confers neuroprotection following SCI (Mills *et al.* 2001b). Since LY 367385 did not affect extracellular EAA release in the current study, it seems unlikely that the antagonist-induced neuroprotective effects of mGluR1 are a result of a

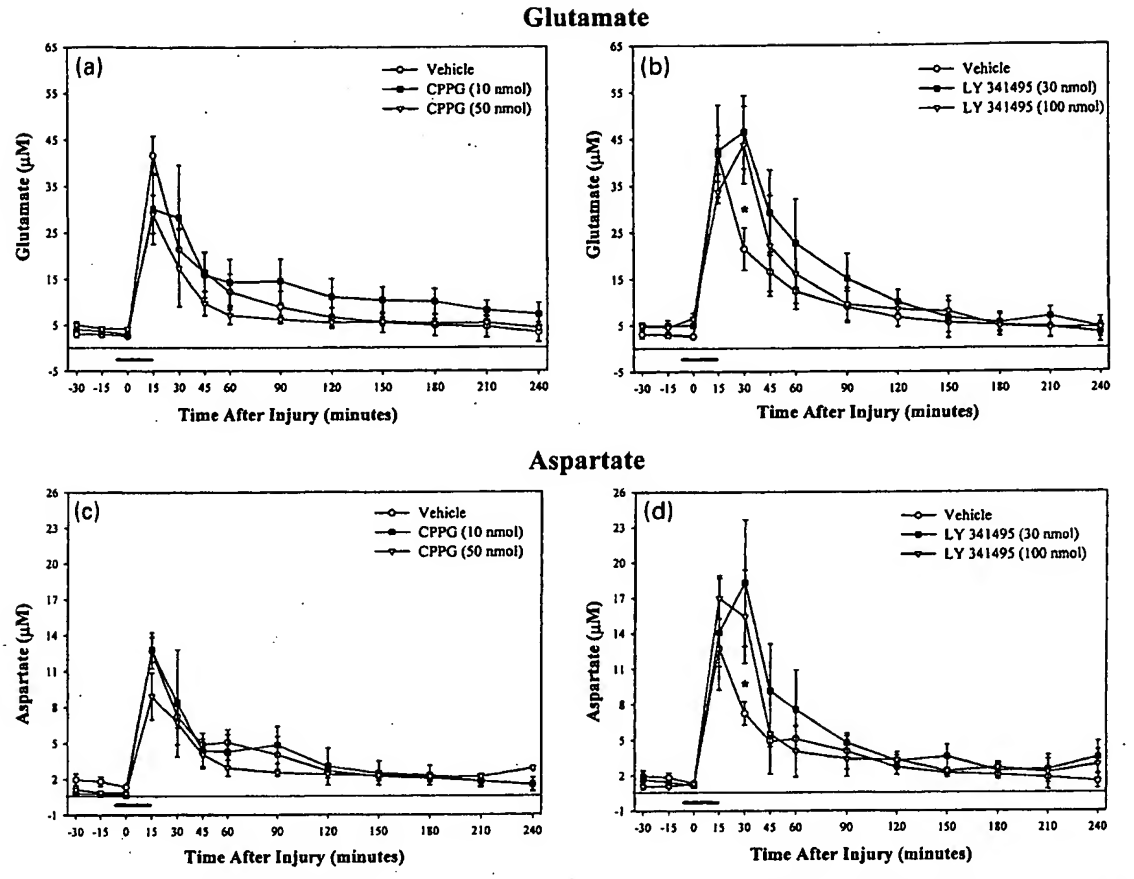

Fig. 7 Time course of extracellular glutamate (a and b) and aspartate (c and d) concentrations after SCI in CPPG- (a and c) and LY 341495-treated (b and d) groups. LY 341495, but not CPPG, increased extracellular EAAs at 30 min following injury. Data are means ± SEM. *Indicates a statistically significant difference between the vehicle and treatment groups ($p < 0.05$). Treatment duration is indicated by a horizontal line above the *x*-axis.

reduction in EAA release following injury. Furthermore, treatment with MPEP at doses that reduce EAA release following SCI does not result in overall tissue sparing (Mills *et al.* 2001b). Since the neuroprotective effects of both mGluR1 and mGluR5 appear to be independent of their effects on EAA release, it may be that inhibition of group-I mGluR-mediated intracellular pathways that lead to cell death (e.g. release of Ca^{2+} from intracellular stores) are responsible for the observed neuroprotective effects following SCI. Similarly, the dose of L-AP4 that reduces extracellular EAA levels in the current study did not produce significant neuroprotection following SCI in a previous study (Mills *et al.* 2001c). Previous reports using *in vitro* models of neuronal trauma indicate that at least some of the neuroprotective effects induced by antagonism of group-I mGluRs (Mukhin *et al.* 1996, 1997) and group-III mGluRs (Faden *et al.* 1997) are independent from mGluR and NMDA receptor interactions. Taken together, these results suggest that modulation of extracellular glutamate concentrations may not be the primary mechanism by which mGluRs influence cell loss after CNS injury. Future experiments exploring this relationship and examining other mechanisms of mGluR mediated cell death following SCI are warranted.

Summary

The current study shows that group-I mGluRs modulate extracellular EAA and GABA levels following SCI. Antagonism of mGluR5 produced a greater reduction in extracellular EAAs than did antagonism to mGluR1 following SCI. When both treatments were combined, a greater reduction in extracellular EAAs was seen than for either individual treatment. Activation of group-III, but not

group-II, mGluRs following SCI reduced extracellular EAA levels without affecting GABA concentrations. Extracellular GABA concentrations followed a similar temporal pattern as the extracellular EAA concentrations. That is, both GABA and EAA levels were maximally increased during the first 15 min of injury. Previous reports show that group-I mGluR antagonists, but not group-II or -III agonists, are neuroprotective following SCI. These neuroprotective effects do not correlate with the effects of these agents on EAA release, suggesting that mGluR-mediated intracellular pathways not directly responsible for modulating neurotransmitter release may help determine cell survivability following CNS injury.

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Group II Metabotropic Glutamate Receptors Modulate Extracellular Glutamate in the Nucleus Accumbens

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ABSTRACT

The regulation of extracellular glutamate in the nucleus accumbens by group II metabotropic glutamate receptors (mGluR2/3) was examined in vivo. Stimulation of mGluR2/3 with 2*R*,4*R*-4-aminopyrrolidine-2,4-dicarboxylate (APDC) or *N*-acetylaspartylglutamate reduced extracellular glutamate levels. Conversely, blockade of mGluR2/3 by LY143495 or (*RS*)-1-amino-5-phosphonoindan-1-carboxylic acid (APICA) increased extracellular glutamate, an effect antagonized by the coadministration of APDC. These effects likely involve both vesicular and nonvesicular glutamate, because the increase in glutamate by APICA or the decrease by APDC was prevented by blocking N-type calcium channels and the release of glutamate after potassium-induced membrane depolarization was antagonized by APDC. In addition, blockade of the cystine-glutamate exchange, a major nonvesicular source of extracellular glutamate, by (*S*)-4-carboxyphenylglycine blocked the effects induced by either APDC or APICA. However, blockade of Na^+ channels by tetrodotoxin or Na^+-dependent glutamate transporters by DL-*threo*-β-benzyloxyaspartate failed to affect the alterations in extracellular glutamate by APICA or APDC, respectively. Group II mGluRs are G_i-coupled and coperfusion with the cAMP-dependent protein kinase (PKA) activator Sp-cAMPS blocked the reduction in glutamate by APDC and the PKA inhibitor Rp-cAMPS prevented the elevation in glutamate by APICA. Taken together, these data support three conclusions: 1) group II mGluRs regulate both vesicular and nonvesicular release of glutamate in the nucleus accumbens, 2) there is tonic in vivo stimulation of mGluR2/3 by endogenous glutamate, and 3) modulation of group II mGluRs of extracellular glutamate is Ca^{2+}- and PKA-dependent.

Metabotropic glutamate receptors (mGluRs) belong to a class of G protein-coupled receptors that is comprised of eight different subtypes that have been organized into three groups based upon sequence homology and coupling to intracellular messengers. Group I receptors (mGluR1,5) are coupled to phospholipase C, whereas group II (mGluR2,3) and group III (mGluR4,6,7,8) receptors are negatively coupled to adenylate cyclase (for review, see Conn and Pin, 1997). Group II and III mGluRs act to inhibit neurotransmitter release both as autoreceptors located on glutamatergic terminals or as presynaptic heteroreceptors. Extensive studies have emerged indicating that mGluRs play an important role in neuroplasticity (Anwyl, 1999), and various drugs targeting group II mGluRs have therapeutic potential including, protection from excitotoxity, treatment of anxiety, Parkinson's disease, schizophrenia, and drug addiction (for review, see Conn and Pin, 1997). A possible role in addiction is indicated by the recently described involvement of glutamate transmission in the nucleus accumbens (NAcc) and the possibility that reducing glutamate transmission by group II mGluR agonists may be of therapeutic benefit (Cornish and Kalivas, 2000; Vanderschuren and Kalivas, 2000).

Group II mGluRs are expressed in the nucleus accumbens (Ohishi et al., 1993a, 1993b; Testa et al., 1998). Selective activation of the group II mGluRs in the NAcc blocks amphetamine-induced locomotor behavior (Kim et al., 2000). In vitro electrophysiological studies in brain slices confirm that group II mGluRs inhibit glutamate release in the NAcc (Manzoni et al., 1997). Moreover, in vivo microdialysis studies show that group II agonists reduce extracellular dopamine in the NAcc (Hu et al., 1999).

Although the presence of group II mGluRs in the NAcc has been established, the identity and the properties of group II mGluRs in modulation of glutamate release remains unclear. For example, the basal level of extracellular glutamate is derived from both vesicular and nonvesicular sources (Timmerman and Westerink, 1997), and it is not known which

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ABBREVIATIONS: mGluRs, metabotropic glutamate receptors; APDC, (2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate; NAAG, *N*-acetylaspartylglutamate; 2-PMPA, 2-(phosphonomethyl) pentanedioic acid; APICA, (*RS*)-amino-5-phosphonoindan-1-carboxylic acid; (*S*)-4CPG, (*S*)-4-carboxyphenylglycine; TTX, tetrodotoxin; TBOA, DL-threo-β-benyloxyaspartate; Sp-/Rp-cAMPS, Sp-/Rp-adenosine 3′5′-cyclic monophosphothioate triethylamine; PKA, cAMP-dependent protein kinase; ANOVA, analysis of variance; PLSD, protected least significant difference; NAcc, nucleus accumbens; NMDA, *N*-methyl-D-aspartate.

glutamate pool is modulated by group II mGluRs. In addition, activation of group II mGluRs has been shown to inhibit cAMP formation in in vitro expression systems, brain slices, and neuronal cultures, but it is unknown whether cAMP signaling is also mediating the effects of group II mGluRs in vivo (for review, see Conn and Pin, 1997). Thus, the present study used in vivo microdialysis combined with mGluR2/3 immunoblotting to characterize the modulation of extracellular glutamate by direct perfusion of various group II selective agonists or antagonists into the NAcc. Experiments were also conducted to examine the involvement of various ion channels, the cystine-glutamate exchanger, glutamate transporters, and the intracellular cAMP/c-AMP-dependent protein kinase (PKA) signaling cascade in mGluR modulation of glutamate release.

Materials and Methods

Animals Housing and Surgery. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The subjects were male Sprague-Dawley rats (Charles Rivers Laboratories, Inc., Wilmington, MA) weighing 250 to 275 g upon arrival and were individually housed in an American Association for Laboratory Animal Care-approved facility maintained on a 12-h light/dark cycle (lights on 7 AM). All experimentation was conducted during the light period. Using ketamine (100 mg/kg) and xylazine (3 mg/kg) anesthesia, dialysis guide cannulae (20 gauge, 14 mm; Small Parts, Roanoke, VA) were implanted over the nucleus accumbens [+1.6 mm anterior to Bregma, ±1.6 mm mediolateral, −4.7 mm ventral to the skull surface according to the atlas of Paxinos and Watson (1986)] using a 6° angle from vertical. The guide cannulae were fixed to the skull with four stainless steel skull screws (Small Parts) and dental acrylic. Surgeries were performed 5 to 7 days after arrival of the subjects, and dialysis experiments were begun 1 week after the surgical procedure.

In Vivo Microdialysis. The night before the experiment, concentric microdialysis probes (with 2 mm of active membrane) were inserted 3 mm beyond tips of guide cannulae into the nucleus accumbens. Dialysis buffer (5 mM KCl, 140 mM NaCl, 1.4 mM $CaCl_2$ 1.2 mM $MgCl_2$, 5.0 mM glucose, plus 0.2 mM phosphate-buffered saline to give a pH of 7.4) was advanced through the probe at a rate of 2 μl/min via syringe pump (Bioanalytical Systems, West Lafayette, IN). Beginning at 2 h after turning on the pump at 8 AM the next morning, baseline samples were collected at 20-min intervals for 100 min. After collecting the baseline samples various drugs were administered via reverse dialysis into the NAcc.

Multiple doses of each mGluR agonist or antagonist were administered alone or in combination with other drugs. Dosage ranges of the various drugs were based upon the relative EC_{50} or IC_{50} values for binding to the respective receptors. *N*-acetylaspartylglutamate (NAAG) was purchased from Sigma-RBI (Natick, MA), and all other mGluR compounds, including (2*R*,4*R*)-aminopyrrolidine-2,4-dicarboxylate (APDC), (*RS*)-1-amino-5-phosphonoindan-1-carboxylic acid (APICA), LY143495, and (*S*)-4-carboxyphenylglycine [(*S*)-4CPG] were purchased from Tocris (Ballwin, MO). NAAG was dissolved with filtered dialysis buffer (see below), whereas all other mGluR compounds were initially dissolved in 0.1 N NaOH (Sigma, St. Louis, MO) and neutralized with 0.1 N HCl (Sigma) to a concentration of 10^{-2} M. Working concentrations were then made by diluting with filtered dialysis buffer. Diltiazem and tetrodotoxin (TTX) were purchased from Tocris, and ω-conotoxin GVIA, Sp- and Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine (Sp-cAMPS, Rp-cAMPS) were obtained from Sigma-RBI. 2-(Phosphonomethyl) pentanedioic acid (2-PMPA) was a gift from Guilford Pharmaceuticals, Inc. (Baltimore, MD) and DL-*threo*-β-benzyloxyaspartate (TBOA) was a gift from Dr. Keiko Shimamoto (Suntory Institute for Bioorganic Research, Osaka, Japan). All of the drugs were dissolved with filtered dialysis buffer and were freshly prepared on day of the experiment. In some experiments KCl was used to increase glutamate release and in these experiments NaCl was reduced proportionally to retain iso-osmolarity.

Quantification of Glutamate. The concentration of glutamate in the dialysis samples was determined using HPLC with flourometric detection. The dialysis samples were collected into 10 μl of 0.05 M HCl containing 2 pmol of homoserine as an internal standard. The mobile phase consisted of 13% acetylnitrile (v/v), 100 mM Na_2HPO_4, and 0.1 mM EDTA, pH 6.04. A reversed-phase column (10 cm, 3 μm ODS; Bioanalytical Systems, West Lafayette, IN) was used to separate the amino acids, and precolumn derivatization of amino acids with *o*-phthalaldehyde was performed using a model 540 autosampler (ESA, Inc., Chelmsford, MA). Glutamate was detected by a fluorescence spectrophotometer (Linear Flour LC 305; ESA Inc.) using an excitation wavelength of 336 nm and an emission wavelength of 420 nm. The area under curve of the glutamate and homoserine peaks was measured with ESA 501 Chromatography Data System. Glutamate values were normalized to the internal standard homoserine and compared with an external standard curve for quantification. The limit of detection for glutamate was 1 to 2 pmol.

$mGluR_{2/3}$ Immunoblotting. To determine the existence of mGluR2/3 proteins in the NAcc, eight rats were decapitated, and the brains were rapidly removed and dissected into coronal sections on ice. The appropriate brain regions were sampled on an ice-cooled Plexiglas plate using a 15-gauge tissue punch, including the prefrontal cortex, parietal cortex, ventral tegmental area, dorsolateral striatum, medial nucleus accumbens (predominately medial shell), and lateral nucleus accumbens (core). Brains punches were immediately frozen on dry ice and stored at −80°C until homogenized for immunoblotting.

The dissected brain punches were homogenized with a handheld tissue grinder in homogenization medium (0.32 M sucrose, 2 mM EDTA, 1% sodium dodecyl sulfate, 50 μM phenyl methyl sulfonyl fluoride, and 1 μg/ml leupeptin, pH 7.2), subjected to low-speed centrifugation (2000*g*, to remove insoluble material) and stored at −80°C. Protein determinations were performed using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Samples (30 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel (8%) electrophoresis utilizing a mini-gel apparatus (Bio-Rad), transferred via semidry apparatus (Bio-Rad) to nitrocellulose membrane, and probed for the proteins of interest (1 gel/protein/brain region). mGluR2/3 was identified using a rabbit anti-rat antibody (1:3000) purchased from Upstate Biotech (Lake Placid, NY) that was made against a peptide containing the C terminus. In control experiments a synthesized peptide having the same 21 amino acid sequence on the C terminus of mGluR2/3 was used to competitively inhibit the binding of antibody to mGluR2/3. Labeled proteins were detected using an horseradish peroxidase-conjugated anti-rabbit secondary IgG diluted 1:30,000 (Upstate Biotech) and visualized with enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL). Assurance of even transfer of protein was evaluated with Ponceau S (Sigma) followed by destaining with de-ionized water. Immunoreactive levels were quantified by integrating band density × area using computer-assisted densitometry (NIH Image version 1.60). The density × area measurements were averaged over three control samples for each gel and all bands were normalized as percent of the control values.

Histology. After the dialysis experiments, rats were administered an overdose of pentobarbitol (>100 mg/kg i.p.) and transcardially perfused with 0.9% saline followed by 10% formalin solution. Brains were removed and placed in 10% formalin for at least 1 week to ensure proper fixation. The tissue was blocked, and coronal sec-

tions (100 μm thick) were made through the site of dialysis probe with a vibratome. The brains were then stained with cresyl violet to verify anatomical placement according to the atlas of Paxinos and Watson (1986).

Statistical Analysis. The StatView statistics package was used to estimate statistical significance. A one-way ANOVA with repeated measures over dose was used to determine the effect of individual drugs on extracellular glutamate levels. A two-way ANOVA with repeated measures over time or dose were used to compare between treatments. Upon identification of statistical significance, post hoc comparisons were made with a Fischer's PLSD.

Results

mGluR2/3 Immunoproteins Are Highly Expressed in the Nucleus Accumbens. A high density of mGluR2/3 immunoproteins were detected in many brain regions including the shell and core of the nucleus accumbens, prefrontal cortex, ventral tegmental area, and striatum of rats. Both dimer and monomer forms were detected, and the dimer was the predominant form of mGluR2/3 in all brain nuclei examined. Figure 1 shows representative immunoblots that illustrate the two forms of mGluR2/3 proteins in the nucleus accumbens (shell and core) and prefrontal cortex. Figure 1 also shows that both the dimer and monomer forms could be completely absorbed by a synthetic peptide having the identical 21 amino acid sequence with the C terminus of mGluR2/3.

Group II mGluRs Reduce Extracellular Glutamate Levels in the Nucleus Accumbens. A selective agonist or antagonist for mGluR2/3 was perfused into the accumbens by reverse microdialysis and the levels of extracellular glutamate were estimated. Figure 2A shows that the mGluR2/3 agonist APDC elicited a dose-dependent decrease in extracellular glutamate levels and this effect was attenuated by the specific group II mGluR antagonist APICA (Fig. 2B). The threshold dose for producing a significant reduction was 5 μM APDC, and the reduction in extracellular glutamate was reversed by washing out the drug with dialysis buffer. Furthermore, NAAG, a mGluR3 agonist (Wroblewska et al., 1997; Schweitzer et al., 2000) elicited a dose-dependent decrease in extracellular glutamate levels in the nucleus accumbens (Fig. 2, C and D). The minimal effective dose of NAAG was 10 μM. The experiment was conducted in the presence of 500 μM 2-PMPA to inhibit the formation of glutamate derived from the metabolism of NAAG by NAALADase (Slusher et al., 1999). In the absence of 2-PMPA the capacity of NAAG to inhibit extracellular glutamate could not be demonstrated (data not shown). 2-PMPA alone did not significantly alter the extracellular levels of glutamate (Fig. 2C).

Conversely, perfusion of the mGluR2/3 antagonist APICA or LY143495 into the nucleus accumbens produced a dose-

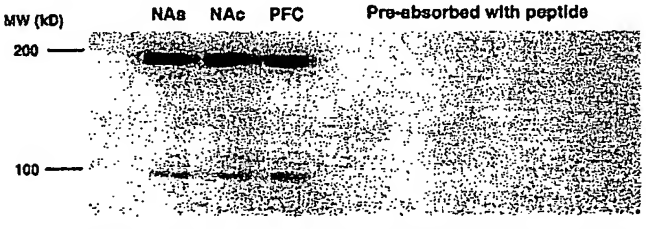

Fig. 1. mGluR2/3 immunoreactivity in the rat brain. The top panel shows representative Western blots demonstrating that two bands (left three lanes) were detected in the shell and core of the nucleus accumbens and the prefrontal cortex (PFC) corresponding to the monomer and dimer of mGluR2/3. Both bands were completely absorbed by a synthetic peptide having the identical 21 amino acids to the C-terminal of mGluR2/3 (right three lanes).

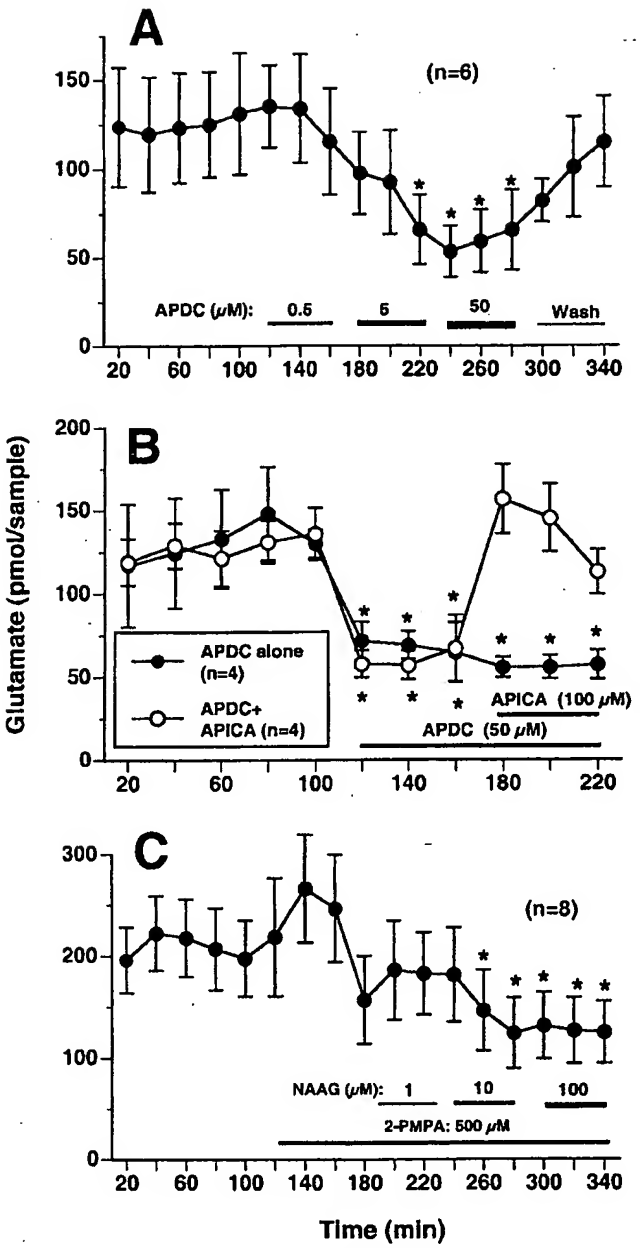

Fig. 2. Group II mGluR agonists decrease extracellular glutamate in the nucleus accumbens. A, reverse dialysis of the mGluR2/3 agonist APDC produced a reversible, dose-dependent decrease in extracellular glutamate levels. One-way ANOVA with repeated measurement over doses reveals that this decrease in glutamate by APDC is significant ($F_{(16,113)} = 6.54$, $p < 0.05$). Each point represents mean ± S.E.M. of picomoles of glutamate per 20-min sample. B, the inhibitory effect of 50 μM APDC was competitively reversed by coadministration of 100 μM APICA, a selective group II antagonist. C, the selective mGluR3 agonist NAAG dose dependently decreased extracellular glutamate in the nucleus accumbens (one-way ANOVA over doses with repeated measurement; $F_{(22,132)} = 54.12$, $p < 0.001$) in the presence of 500 μM 2-PMPA, to inhibit NAAG degradation by NAALADase. *, $p < 0.05$, compared with the average of the last three of the five baseline samples using a Fisher PLSD for post hoc comparisons. n = XX on each figure indicates animal number in each group of study.

dependent increase in extracellular glutamate (Fig. 3). Further the increase by APICA was reversed by coperfusion of the group II agonist APDC (Fig. 3B). The threshold dose for a significant response by APICA and LY143495 was 10 μM and 10 nM, respectively, and drug washout with dialysis buffer reversed the increase by the highest dose of APICA (1 mM).

mGluR2/3 Modulation of Extracellular Glutamate is Ca^{2+}-Dependent. Basal extracellular glutamate derives from both neuronal and glial sources, and can be derived from vesicular or cytoplasmic pools (Timmerman and Westerink, 1997). Vesicular neurotransmitter release by high K^+ is predominantly Ca^{2+}-dependent (for review, see Timmerman and Westerink, 1997). To determine whether the reduction in extracellular glutamate by the group II mGluR agonists is derived from vesicular stores of glutamate, the capacity of APDC to reverse the release of glutamate by a high concentration of K^+ (80 mM) was examined. Figure 4, A and B, illustrate that the high K^+-evoked glutamate release was significantly inhibited by the coadministration of 50 μM APDC. In further support of a role for Ca^{2+}-dependent vesicular release of glutamate, either the L- or N-type Ca^{2+} channel blockers diltiazem or ω-conotoxin GVIA, respectively, was coinfused into the NAcc with APICA. Either drug completely blocked the elevation of extracellular glutamate produced by APICA (Fig. 5A). Whereas diltiazem alone had no significant effect, ω-conotoxin GVIA alone significantly reduced the basal level of extracellular glutamate by 30 to 40%. Furthermore, coadministration of ω-conotoxin GVIA blocked the capacity of APDC to reduce extracellular glutamate (Fig. 4B). These data suggest that the reduction in basal extracellular glutamate by N-type Ca^{2+} channel blockade and mGluR2/3 stimulation were not additive and may involve the same or overlapping mechanisms.

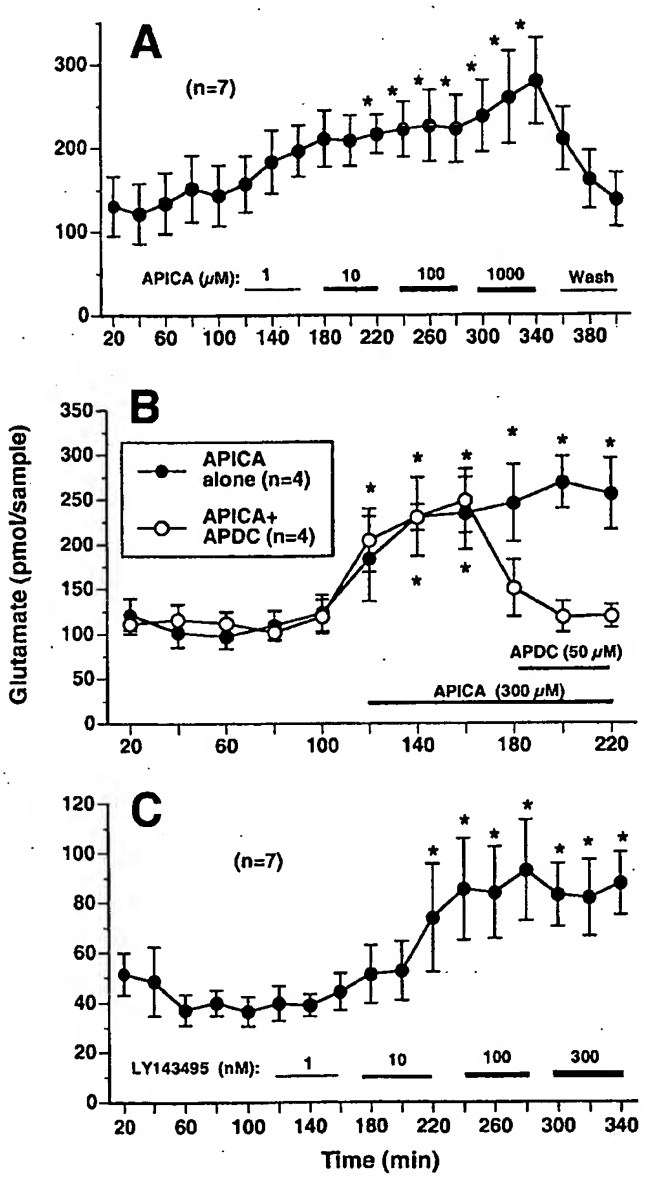

Fig. 3. Group II mGluR antagonists elevate extracellular glutamate in the nucleus accumbens. A, administration of the group II mGluR antagonist APICA dose dependently increased extracellular glutamate levels over the entire dose range tested ($F_{(16,64)} = 6.87$, $p < 0.001$). B, the increase in extracellular glutamate by 300 μM APICA was blocked by coadministration of 50 μM APDC. C, LY143495, another highly potent, selective group II antagonist, dose dependently increased extracellular glutamate ($F_{(16,85)} = 9.74$, $p < 0.05$). *, $p < 0.05$, compared with the average of the last three of the five baseline samples using a Fisher PLSD for post hoc comparisons.

In contrast to the involvement of extracellular Ca^{2+}, pretreatment with the voltage-dependent Na^+ channel blocker TTX at a dose sufficient to nearly eliminate detectable extracellular levels of monoamine transmitters (1 μM; Timmerman and Westerink, 1997) did not block the dose-dependent increase in extracellular glutamate elicited by APICA (Fig. 5C). TTX alone did not significantly alter the basal concentration of glutamate. This result argues that mGluR2/3 directly regulates Ca^{2+}-dependent release of glutamate and is not acting indirectly via a *trans*-synaptic mechanism. Although Na^+-dependent glutamate transporters play an important role in modulating the basal level of extracellular glutamate, blockade of glutamate uptake by TBOA, a broad-spectrum glutamate uptake inhibitor (Shimamoto et al., 1998), did not attenuate the APDC-induced reduction in glutamate (Fig. 5D), suggesting that the effect of mGluR2/3 stimulation on extracellular glutamate is independent of glutamate transporters.

mGluR2/3 Involves Cystine-Glutamate Exchange. The basal level of extracellular glutamate measured by microdialysis is predominantly controlled by cystine-glutamate exchange, which provides the primary source of extracellular, nonvesicular glutamate (Baker et al., 2001). To determine whether group II mGluRs might reduce extracellular glutamate by negatively modulating cystine/glutamate exchange, the inhibitor of cystine/glutamate exchange (*S*)-4CPG (Ye et al., 1999) was infused into the NAcc. Coinfusion of (*S*)-4CPG with APICA or APDC prevented the increase in glutamate by APICA or the decrease by APDC (Fig. 6, A and B). (*S*)-4CPG (5 μM) alone decreased extracellular glutamate by approximately 50% (Fig. 6A).

Signaling through PKA Mediates Group II mGluR Reduction in Extracellular Glutamate. Group II mGluRs are negatively coupled to adenylate cyclase and PKA via inhibitory G_i proteins (Conn and Pin, 1997; Anwyl, 1999). To evaluate a role for PKA in the capacity of mGluR2/3 to modulate extracellular glutamate levels, the PKA activator Sp-cAMPS or the PKA inhibitor Rp-cAMPS

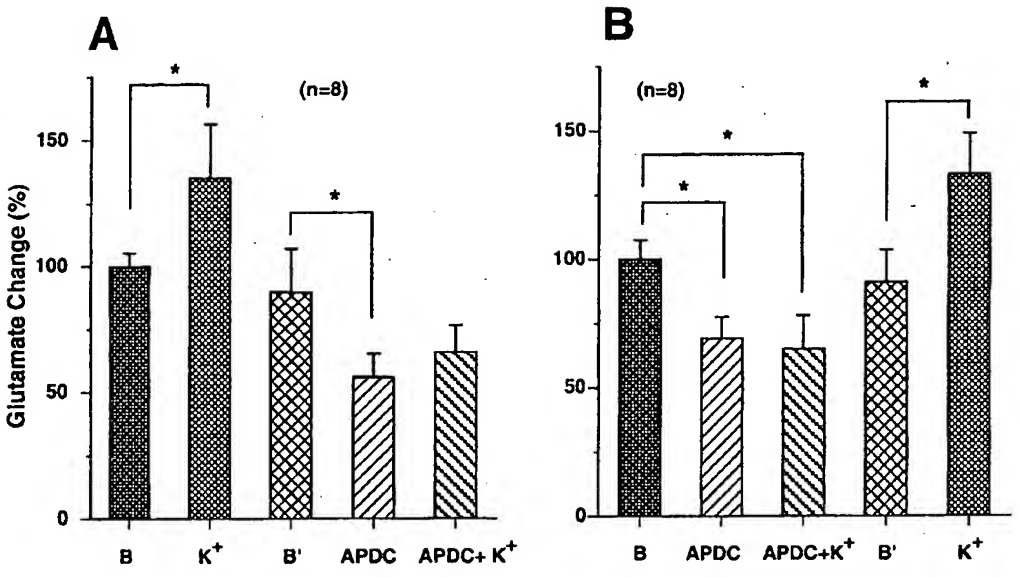

Fig. 4. The group II mGluR agonist 50 μM APDC blocked 80 mM K^+-stimulated glutamate release in the nucleus accumbens. Each panel is a separate experiment giving the same drug treatment in a different sequence to verify that the drug would wash out and not produce enduring impairment of K^+-evoked glutamate release. A one-way ANOVA with repeated measures over time (each drug treatment) indicated that K^+ stimulation and APDC significantly altered the basal level of extracellular glutamate (*A*, $F_{(4,39)} = 13.31$, $p < 0.001$; *B*, $F_{(4,39)} = 4.23$, $p < 0.005$). *, $p < 0.05$, compared with the average of each baseline (B or B') before the drug administration.

was perfused into the accumbens via the dialysis probe in combination with the mGluR2/3 agonist APDC or the antagonist APICA. Figure 7, A and C show the effect of increasing doses of Sp-cAMPS or Rp-cAMPS alone. Although Sp-cAMPS elevated glutamate levels at lower doses and decreased levels at higher doses, Rp-cAMPS reduced glutamate at lower doses and increased levels at higher doses. Based upon these dose-response curves a relatively low dose of each drug (5 nM) was coadministered with APDC or APICA. Figure 7B shows that Sp-cAMPS attenuated the APDC-induced decrease in extracellular glutamate. Conversely, Rp-cAMPS inhibited APICA-induced increase in extracellular glutamate (Fig. 7D). The inhibitory effect of both Sp-cAMPs and Rp-cAMPS were reversible because after wash-out with dialysis buffer, the capacity of APDC to reduce and APICA to elevate extracellular glutamate was restored.

Histology. Figure 8 depicts the dialysis probe placements in the nucleus accumbens. The majority of probe placements in the nucleus accumbens were at or medial to the anterior commissure. Placements tended to be primarily in the core of the nucleus accumbens, although a number were located at the interface between the core and either the medial or the ventral limb of the shell, and a minority of placements were primarily in the shell. In addition, some probes were partly (<30%) dorsal to the nucleus accumbens in the striatum or septal region.

Discussion

These data provide in vivo evidence that pharmacological stimulation of group II mGluRs in the nucleus accumbens reduces the basal concentration and K^+-evoked increases in extracellular glutamate. Moreover, group II mGluRs bear significant endogenous tone because blockade of mGluR2/3 elevates extracellular glutamate levels. The effects of the group II compounds were shown to require active L- and N-type Ca^{2+} conductances, as well as functional cystine-glutamate exchange, and to be signaled through cAMP/PKA cascade. In contrast, there was no role identified for voltage-dependent sodium channels or glutamate transporters.

Group II mGluRs Act as Autoreceptors to Inhibit Presynaptic Glutamate Release in the Nucleus Accumbens. In vitro electrophysiological experiments have revealed that a prominent physiological effect of mGluR2/3 agonists in the cortex and hippocampus is to reduce glutamatergic transmission by stimulating presynaptic autoreceptors (Anwyl, 1999), which has been confirmed as well in studies examining in vitro glutamate release (Cartmell and Schoepp, 2000). Although two in vivo studies have revealed the capacity of systemically administered mGluR2/3 agonist to reduce evoked glutamate release in the prefrontal cortex and striatum (Battaglia et al., 1997; Moghaddam and Adams, 1998), the present study is the first in vivo demonstration that locally stimulating group II mGluRs lowers extracellular glutamate. Moreover, the in vivo measurements revealed the presence of substantial tone by endogenous glutamate on mGluR2/3 in the nucleus accumbens. Thus, blocking mGluR2/3 elevated extracellular glutamate, and consistent with an action on presynaptic glutamate terminals, the increase was blocked by L- and N-type Ca^{2+} channel antagonists, but not by blocking voltage-dependent Na^+ channels. Although N- and P/Q-types of Ca^{2+} channels are thought to mediate vesicular glutamate release from nerve terminals (Anwyl, 1991, 1999), L channels are predominantly located on soma, dendrites, and/or glial cells (Anwyl, 1999; Nachman-Clewner et al., 1999). Diltiazem blocked the APICA-induced increase in glutamate, suggesting that the L-type Ca^{2+} channels and mGluR2/3 on somatodendrites or glial cells, rather than just presynaptic mGluR2/3, are playing a role in modulating vesicular and/or nonvesicular glutamate release. Also consistent with a presynaptic site of action is the inhibition of the K^+-mediated release of glutamate by APDC.

To further determine the involvement of the subtypes of group II mGluRs, the effect of the mGluR3 agonist NAAG (Wroblewska et al., 1997; Schweitzer et al., 2000) was examined. NAAG decreased extracellular glutamate levels in the

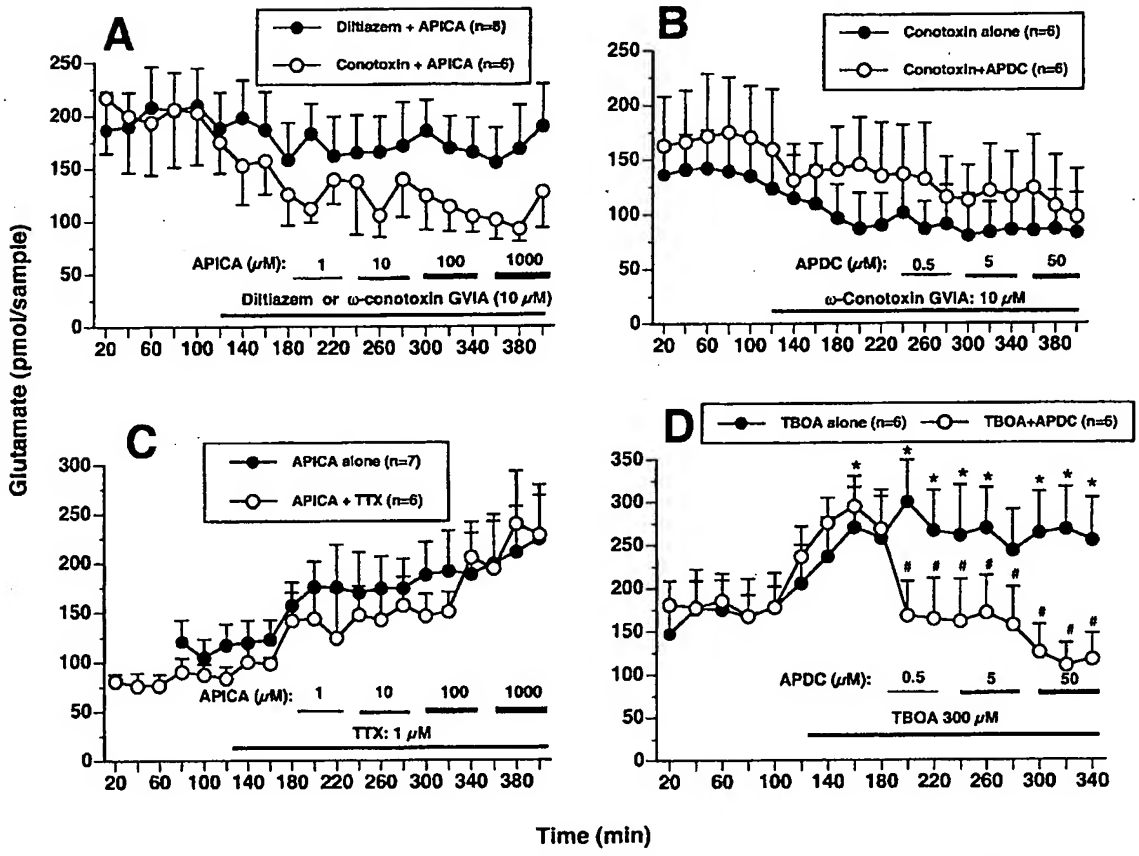

Fig. 5. Involvement of calcium channels, sodium channels, or glutamate transporters in mGluR2/3 regulation of extracellular glutamate. A, coadministration of L- and N-type Ca^{2+} channel blockers diltiazem (10 μM) and ω-conotoxin GVIA (10 μM), respectively, blocked APICA-induced increase in extracellular glutamate. After collecting five baseline samples the Ca^{2+} channel antagonists were introduced into the dialysis buffer for the remainder of the experiment as indicated by the bars. A one-way ANOVA with repeated measurement over the entire dose range indicates that APICA did not increase extracellular glutamate in the presence of either diltiazem or ω-conotoxin GVIA. B, N-type Ca^{2+} channel blocker ω-conotoxin GVIA antagonized the APDC-induced decrease in glutamate. A two-way ANOVA with repeated measures over time indicates that the extracellular glutamate level was significantly decreased over time (drug treatment) ($F_{(19,190)} = 4.5$, $p < 0.001$), but no significant treatment × time interaction ($F_{(19,190)} = .35$, $p > 0.05$) was observed. ω-Conotoxin GVIA alone decreased the basal level of extracellular glutamate by around 30 to 40% beginning at 60 min after introduction into the dialysis buffer ($F_{(19,119)} = 3.39$, $p < 0.001$). C, coperfusion of the Na^+-channel blocker tetrodotoxin (TTX, 1 μM) failed to block the APICA-induced increase in extracellular glutamate. A two-way ANOVA with repeated measures over doses (time) revealed a significant increase in extracellular glutamate ($F_{(12,120)} = 3.87$, $p < 0.05$), but no significant treatment × time interaction ($F_{(12,120)} = .39$, $p > 0.05$). TTX alone had no significant effect on the basal level of extracellular glutamate. D, blockade of glutamate transporters with TBOA failed to alter the inhibitory effect of APDC in glutamate. A two-way ANOVA with repeated measures over time reveals a significant drug effect ($F_{(16,160)} = 4.1$, $p < 0.001$) and treatment × time interaction ($F_{(16,160)} = 5.13$, $p < 0.001$). *, $p < 0.05$, compared with the average of the last three baseline samples. #, $p < 0.05$, comparing TBOA+APDC with TBOA alone at each collection time.

presence of 2-PMPA, an enzyme (NAALADase) inhibitor that prevented NAAG metabolism to glutamate (Slusher et al., 1999). These data indicate that the mGluR3 contributes to the decrease in glutamate by APDC. However, no selective mGluR3 antagonist was available to further verify the role of mGluR3 in modulating endogenous glutamate release. Also, it was reported that NAAG is only 10-fold more selective for mGluR3 than mGluR2 (Cartmell et al., 1998; Schweitzer et al., 2000). Although NAAG may also acts as a weak NMDA agonist, the inhibitory effect of NAAG on glutamate release is unlikely mediated by activating NMDA receptors because previous studies have shown that NMDA receptor activation increases glutamate release in the striatum (Hashimoto et al., 2000).

Group II mGluRs Decrease in Extracellular Glutamate May Involve Cystine-Glutamate Exchange. The cystine-glutamate exchanger is a major nonvesicular source of glutamate. This exchanger is driven by the relative intra- and extracellular substrate gradients and typically operates to transport glutamate out and cystine into the cell (Kato et al., 1993; Warr et al., 1999). The cystine-glutamate exchanger was recently cloned and is found in a variety of tissue types, indicating that it is a primary metabolic source of intracellular cystine. Elevation of extracellular cystine concentration increased glutamate release from brain slices (Warr et al., 1999), an effect that was blocked by the relatively selective cystine-glutamate exchanger inhibitor (*S*)-4CPG (Ye et al., 1999). More recently, Baker et al. (2001) used in vivo microdialysis to show that the basal, extracellular glutamate content is derived mainly from cystine-glutamate exchange, because blockade of the cystine-glutamate exchanger by homocysteic acid or (*S*)-4CPG lowered extracellular glutamate levels by 60 to 70%. In the present study, pretreatment with (*S*)-4CPG prevented the increase in basal extracellular glutamate by APICA or the decrease by APDC. The former action re-

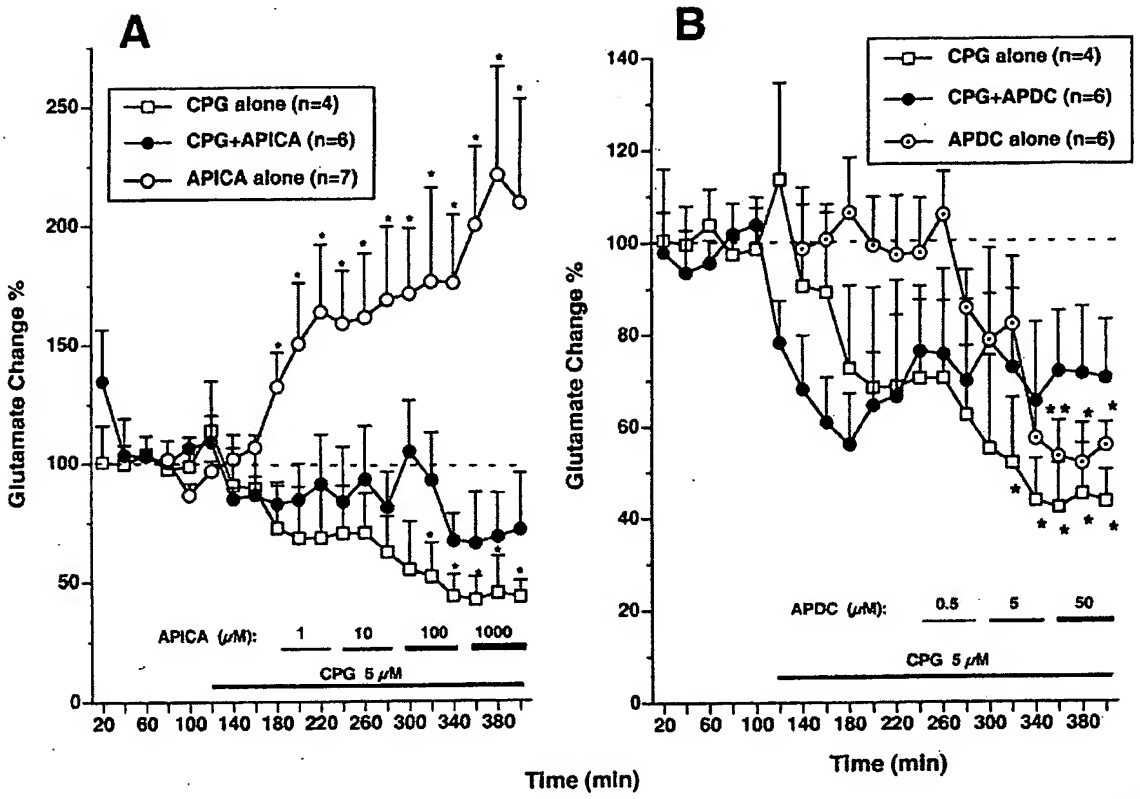

Fig. 6. Group II mGluRs inhibit cystine/glutamate exchanger. A, coadministration of the cystine/glutamate exchanger (*S*)-4CPG (5 μM) blocked the APICA-induced increase in extracellular glutamate. APICA alone dose dependently increases ($F_{(2,17)} = 6.22$, $p < 0.05$), whereas 5 μM (*S*)-4CPG alone significantly lowered extracellular glutamate levels ($F_{(5,17)} = 5.08$, $p < 0.05$). A one-way ANOVA with repeated measurement over the entire dose range indicates that APICA did not increase extracellular glutamate in the presence of (*S*)-4CPG. B, APDC dose dependently decreased extracellular glutamate ($F_{(5,13)} = 4.15$, $p < 0.05$), which was prevented in the presence of 5 μM (*S*)-4CPG. A one-way ANOVA with repeated measures over time indicates that APDC did not decrease extracellular glutamate in the presence of the (*S*)-4CPG. ⋆, $p < 0.05$, compared with the average of the last three baseline samples in each experimental group.

flects decreased glutamate tone by inhibiting cystine-glutamate exchange (Baker et al., 2001), while the latter suggests that cystine-glutamate exchange, at least in part, mediates the action of the group II mGluRs. Although (*S*)-4CPG acts as a group I mGluR antagonist, blockade of group I mGluRs does not alter extracellular glutamate (Baker et al., 2001). The mechanism by which mGluR2/3 may couple to the cystine-glutamate exchanger is unclear. However, the reversal of mGluR2/3 effects on extracellular glutamate by modulating PKA activity indicates that mGluR2/3 inhibition of PKA may be signaling changes in cystine-glutamate exchange.

PKA- and Calcium-Dependent Effects by Group II mGluRs. The most well-characterized signaling event for group II mGluRs is G_i-coupled reductions in cAMP formation and the subsequent inhibition of PKA (for review, see Conn and Pin, 1997). Electrophysiological studies demonstrate that activation of the adenylate cyclase cascade increases glutamatergic transmission in striatum and hippocampal slices, and may be critical in some forms of long-term potentiation (Colwell and Levine, 1995; Trudeau et al., 1996). Furthermore, Chavis et al. (1998) showed that activation of the cAMP/PKA cascade enhances presynaptic vesicle recycling at cerebellar granule cells. Consistent with a role for this signaling cascade in the present study, coperfusion of the selective PKA activator Sp-cAMPS blocked APDC-induced inhibition of glutamate release, whereas the selective PKA inhibitor Rp-cAMPS antagonized APICA-induced increase in extracellular glutamate.

Group II mGluRs have been previously shown to be G_i-coupled, negative modulators of L- and N-type Ca^{2+} channels in electrophysiological studies using brain slices, neuronal cultures, and heterologous expression systems (Chivas et al., 1994; Schumacher et al., 2000). Similarly, in vitro and in vivo release studies reveal L- and N-type channel involvement in the inhibition of dopamine release by group II mGluRs (Hu et al., 1999). In the present study, we observed that coadministration of the Ca^{2+} channel antagonists diltiazem (L-type) or ω-conotoxin GVIA (N-type) abolished the capacity of the group II antagonist to elevate or the agonist to reduce extracellular glutamate. Surprisingly, one electrophysiological investigation reported that blockade of N-type Ca^{2+} channels did not prevent the inhibition of the glutamate transmission induced by group II agonists in the nucleus accumbens (Manzoni et al., 1997). Possible reasons for the distinction from the present study include the use of the less selective mGluR2/3 agonist (2*S*,1′*S*,2′*S*)-2-(2′carboxy-3′,3′-difluorocyclopropyl)glycine and the fact that in the present study N-type channel blockade was maintained for 1 h before administering APDC, which may have resulted in a more complete blockade of the channels. Finally, phosphorylation of presynaptic proteins by

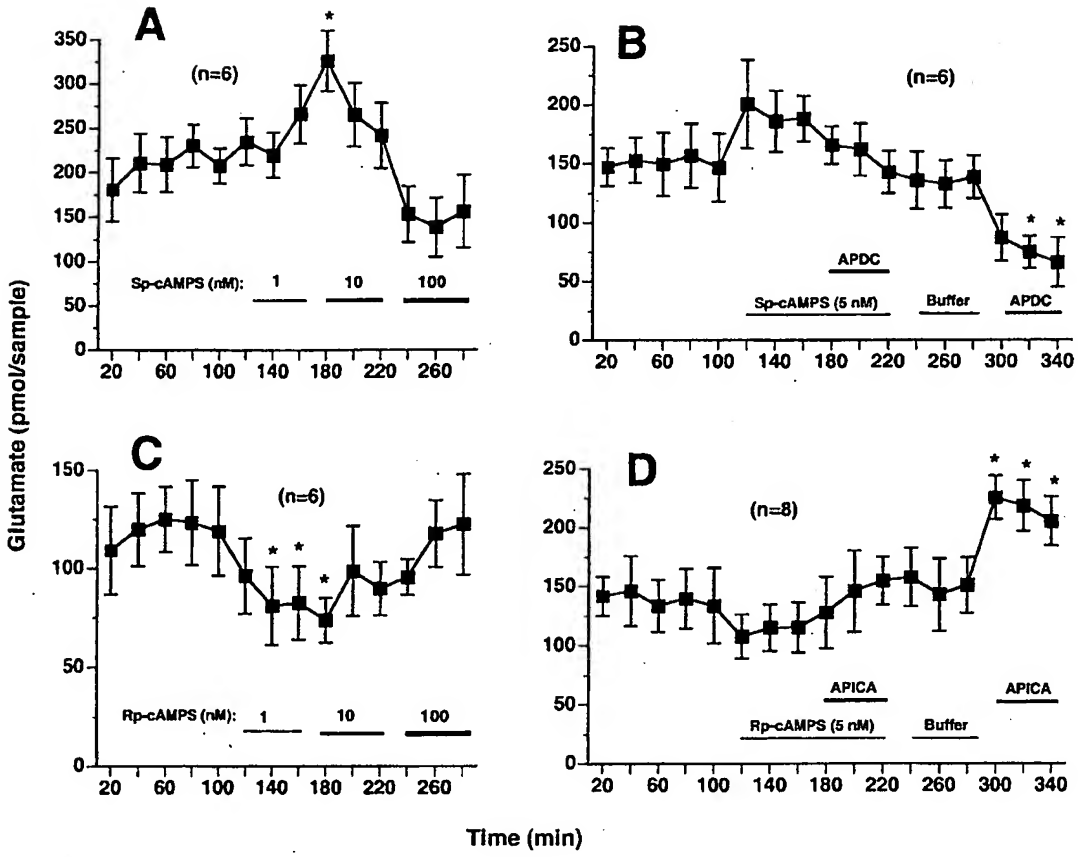

Fig. 7. Intracellular cAMP/PKA cascade mediates group II mGluR modulation of extracellular glutamate. A, dose-dependent effects of Sp-cAMPS, a cAMP-dependent protein kinase activator (one-way ANOVA with repeated measures over dose; $F_{(22,137)} = 3.37$, $p < 0.001$). Lower doses (1–10 nM) of Sp-cAMPS increased, whereas higher doses (10–1000 nM) decreased basal extracellular glutamate in the NAcc. B, coadministration of 5 nM Sp-cAMPS significantly reduced the capacity of APDC to decrease extracellular glutamate ($F_{(16,67)} = 2.35$, $p < 0.005$). C, lower doses (1–100 nM) of Rp-cAMPS (a PKA inhibitor) decreased extracellular glutamate in the NAcc ($F_{(16,84)} = 2.55$, $p < 0.05$). D, Rp-cAMPS (5 nM) blocked the APICA-induced increase in extracellular glutamate. The second APICA (100 μM) administration produced a significant increase in glutamate 1 h later in the same rats ($F_{(16,67)} = 4.10$, $p < 0.001$). *, $p < 0.05$, compared with the average of the last three baseline samples (A and C) or the average of the three samples before APDC or APICA administration.

PKA is known to enhance transmitter release (Greengard et al., 1993) and could contribute to the Ca^{2+}-independent regulation of glutamate transmission by mGluRs observed in some studies (Scanziani et al., 1995).

Dimerization of Group II mGluR Immunoreactive Proteins Detected in Rat Brain. Previous anatomical studies have shown the existence of group II mGluR mRNA in the NAcc (Ohishi et al., 1993a, 1993b; Testa et al., 1998). The present study showed that there is a high density of mGluR2/3 immunoreactive proteins in the NAcc. The majority of mGluR2/3 in the NAcc, as well as in the prefrontal cortex, dorsal striatum and the ventral tegmental area appeared as a dimer. However, it is not known whether the dimer is a homodimer of mGluR2 or mGluR3 or a heterodimer of mGluR2/3, nor is the functional consequence of dimerization understood. Reports of hetero- and homodimerization of a variety of metabotropic receptors have emerged, and the functional consequences of dimerization that have been elucidated are generally consistent with promoting metabotropic receptor trafficking and signaling. For example, the hetero-dimer of GABAB receptor subtypes promotes the trafficking of active GABAB receptors into the plasmallemal membrane (Kuner et al., 1999), and the dimerization of δ-opioid receptors stabilizes receptors in the membrane (Cvejic and Devi, 1997).

In addition to acting as autoreceptors on glutamatergic presynaptic terminals, group II mGluRs are also expressed by astrocytes (Wroblewska et al., 1998). Recent evidence reveals that glia cells can release glutamate in a Ca^{2+}-dependent fashion using presynaptic protein assemblies similar to neuronal synaptic transmission (Araque et al., 1998). Moreover, mGluR receptors can activate Ca^{2+} currents in astrocytes, although most studies attribute this action to group I mGluR stimulation (Bernstein et al., 1998). Given that Ca^{2+}-dependent release of glutamate can occur in both glia and neurons, the in vivo estimates of extracellular glutamate in the present report cannot distinguish effects of mGluR2/3 agonists on neurons versus glia. Similarly, cystine-glutamate exchangers are present in both glia and neurons. Although the lack of effect by TTX supports a primary action on glia (which lack TTX-sensitive sodium channels), the sensitivity of the mGluR effects on glutamate to N-type Ca^{2+} channel blockade supports a role for neurons because N-type channels are absent or in very low abundance in glia (Araque et al., 2000).

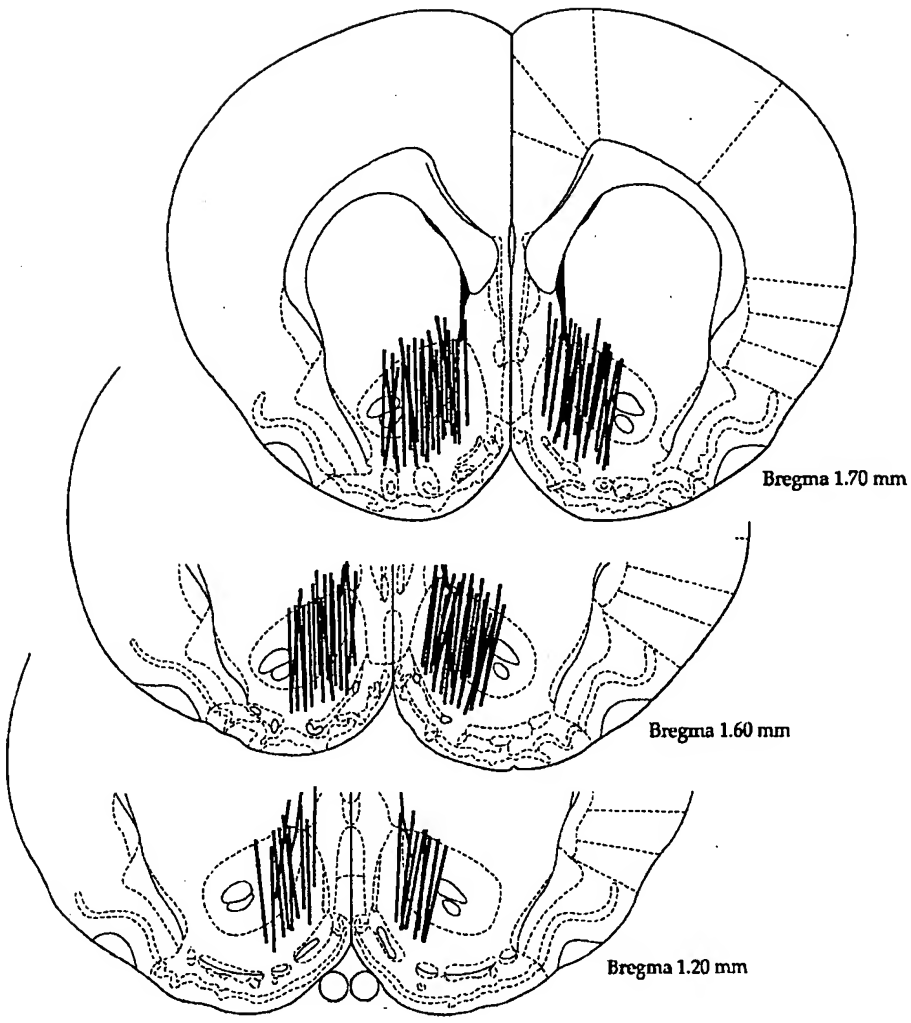

Fig. 8. Location of the microinjection cannula tips in the nucleus accumbens for microdialysis studies. The coronal drawings of the rat brain are based on the atlas of Paxinos and Watson (1986). The lines approximate the location of the 2 mm of active dialysis membrane in the nucleus accumbens.

Conclusions. Group II mGluRs were found to decrease both the Ca^{2+}-dependent vesicular release of glutamate and to involve the cystine-glutamate exchange, a main nonvesicular glutamate source. Moreover, the reduction in extracellular glutamate by stimulating mGluR2/3 was mediated by inhibiting PKA. Importantly, mGluR2/3 were found to bear significant in vivo glutamatergic tone because blocking mGluR2/3 elevated extracellular glutamate levels. This latter finding indicates that the extracellular pool of glutamate measured by microdialysis may regulate glutamate neurotransmission.

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Rapid communication

Glutamate release inhibiting properties of the novel $mGlu_5$ receptor antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP): complementary in vitro and in vivo evidence

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Abstract

We have previously demonstrated that neuronal release of the excitatory amino acid glutamate is facilitated by the selective activation of presynaptic Group I metabotropic autoreceptors. Here we report the release *inhibiting* actions of the novel $mGlu_5$ receptor-selective antagonist, 2-methyl-6-(phenylethynyl)-pyridine (MPEP), both in vitro and in vivo. These data provide compelling evidence for the presence of functional positive modulatory $mGlu_5$ subtype autoreceptors in the mammalian central nervous system.

Keywords: Glutamate; Metabotropic glutamate receptors; Autoreceptors; Presynaptic; MPEP

Metabotropic glutamate (mGlu) receptors comprise a family of eight GTP-binding protein (G-protein)-linked receptors which are subdivided into three groups based on their amino acid sequence homology, signal transduction mechanisms and pharmacological properties (Conn and Pin, 1997). Group I receptor subtypes ($mGlu_1$, $mGlu_5$ and their splice variants) are selectively activated by (S)-3,5-dihydroxyphenylglycine ((S)-3,5-DHPG) and stimulate phospholipase C (PLC), increasing phosphoinositide hydrolysis and diacylglycerol (DAG) generation. Group II ($mGlu_2$ and $mGlu_3$) and Group III ($mGlu_4$ and $mGlu_{6-8}$) mGlu receptors are both negatively coupled to adenylate cyclase but can be distinguished pharmacologically by the use of selective agonists and antagonists (see Schoepp et al., 1999). Herrero and coworkers (1992) provided the first evidence for the existence of central mGlu autoreceptors which facilitate neuronal glutamate release with the demonstration that 4-aminopyridine-evoked glutamate release from rat cerebrocortical synaptosomes can be enhanced by (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3R)-ACPD), a non-selective mGlu receptor agonist. We have recently demonstrated that these presynaptic receptors are of the Group I-type in the rat forebrain slice preparation (Thomas et al., 2000) and in the rat corpus striatum in vivo (Patel and Croucher, 1998). These functional studies also suggested that the receptors mediating this response are unlikely to be of the $mGlu_1$ subtype as antagonists selective for these receptors were unable to influence the (S)-3,5-DHPG-evoked enhancement of electrically-stimulated [^{3}H]D-aspartate ([^{3}H]D-asp) release from rat forebrain slices. However, definitive evidence for the specific mGlu receptor subtype involved has been lacking due to the paucity of high-affinity selective ligands for the mGlu receptor subtypes. We now report the inhibitory actions of the recently described, potent and highly selective, non-competitive $mGlu_5$ receptor antagonist, 2-methyl-6-(phenylethynyl)-pyridine (MPEP) (Gasparini et al., 1999) against (S)-3,5-DHPG-induced release of [^{3}H]D-asp in depolarised rat forebrain slices. The actions of this novel antagonist on glutamate release in vivo, as measured by intracerebral microdialysis, is also reported for the first time.

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A detailed account of the brain slice methodology utilised is given elsewhere (Patel and Croucher, 1997). Briefly, six coronal hemisections (500 μM thick) were obtained from the brains of male Wistar rats (240±10 g; Charles River) and following a 45 min equilibration period in oxygenated brain modified Krebs bicarbonate buffer, these were "loaded" with [^{3}H]D-asp (Amersham International) by individual incubation at 37°C for 45 min in 5 ml of buffer containing 40 nM [^{3}H]D-asp (specific activity 25 Ci/mmol). Each hemisection was then transferred to a gold microelectrode superfusion chamber maintained at 37°C and superfused with oxygenated Krebs buffer at a rate of 0.4 ml/min for 45 min to wash off excess radiolabel. Superfusate samples were subsequently collected every 2 min. Basal superfusate samples were collected for 12 min before a 5 min period of electrical stimulation, comprising biphasic rectangular pulses (36 mA, 2 ms) at a frequency of 20 Hz, was delivered to the tissue. These parameters have previously been shown to provide a sub-maximal, highly Ca^{2+}-dependent, presynaptic $GABA_B$ receptor-regulated, electrically-evoked release of [^{3}H]D-asp from rat forebrain slices (Patel and Croucher, 1997; Thomas et al., 2000). Samples were collected for a further 17 min before (S)-3,5-DHPG (0.1–3.0 μM) or MPEP (0.1–10 μM), alone or in combination, were added to the superfusate. Further basal samples were then collected over a 10 min period before a second period of electrical stimulation, with the same parameters as before, was applied to the tissue. Scintillation fluid was added to the collected superfusates and the radioactive content was quantified by liquid scintillation spectrometry. Mean basal efflux levels (B1 and B2) were calculated as the mean level of radioactivity in the 4 samples immediately preceding the respective periods of electrical stimulation. Responses to electrical stimulation (S1 and S2) were calculated as the total levels of stimulated efflux minus mean basal levels. Ratios of basal (B2/B1) and electrically-stimulated (S2/S1) efflux of label before and after drug application were calculated to assess the influence of the drugs on basal and stimulated release. Patel and co-workers (2001) have previously described in detail the construction and bilateral implantation of microdialysis probes into the striatum of rats for the procedure of in vivo microdialysis. In the following experiments, dialysis monoprobes (3 mm active length; Cuprophan membrane, 350 μM diameter; M.W. cut-off 40 kD) were inserted bilaterally via previously implanted guide cannulae to lie in the corpus striatum (tip position Fr, +1.5; Tr, ±2.5; V, +6.0 relative to bregma) of male Wistar rats (275–300 g; Charles River). The technique for monitoring the efflux of isotopically labelled glutamate, preloaded in the brain in vivo by reverse dialysis, was first described and characterised by Young and co-workers (1990) and, with minor modifications, this methodology was adopted in the following study. All experiments were performed in freely moving animals, over a 2 day period, 24 hours following probe implantation. Animals were connected for dialysis perfusion with artificial cerebrospinal fluid (aCSF) at a flow rate of 3 μl/min for an initial 60 min equilibration period. The tissue was then preloaded with L-3,4-[^{3}H]glutamate ([^{3}H]L-glu; NEN Ltd) by perfusing with aCSF containing 5 μM [^{3}H]L-glu (specific activity 41.1 Ci/mmol) at a reduced flow rate of 1 μl/min for 45 min. Dialysis perfusion was continued with normal aCSF for a further 45 min at 3 μl/min to wash off excess radiolabel from around the perfusion site and then at 7.5 μl/min for the remainder of the experiment. Following the washout period (time=0) samples were collected at consecutive 15 min time-points and scintillation fluid was added to 100 μl aliquots of each sample before quantification of the radioactivity content by liquid scintillation spectrometry. Using the current protocol, the majority of label collected in the dialysate samples, under both basal and stimulated conditions, is known to remain associated with L-glutamate (Young et al., 1990; Young and Bradford, 1993). To confirm the viability of the tissue being perfused, 100 mM potassium (K^+) was applied for 15 min by reverse dialysis through the monoprobes into the striatum at sample points 90 and 240 min. (RS)-3,5-Dihydroxyphenylglycine ((RS)-3,5-DHPG), at dialysate concentrations of 0.1–100 μM, was applied to the striatum at time point 165 min for 15 min, whilst MPEP, 10 μM, was applied at time point 150 min for a 30 min period. The radioactivity content of the samples after high-K^+ stimulation, agonist, or agonist/antagonist combinations was calculated as a maximum percentage change with respect to the preceding, baseline sample. When antagonist alone was tested, the average radioactivity content of the 2 samples over which it was applied to the tissue was taken and the percentage change in radiolabel release was calculated with respect to the preceding drug-free sample. All results are presented as the mean±SEM of n independent observations. Statistical significance of differences in responses was determined using analysis of variance (ANOVA) and a Student's post hoc t-test for independent groups. Values are considered to be significantly different from control if $P<0.05$. All of the above experiments were performed in accordance with the regulations detailed in the Animals (Scientific Procedures) Act, 1986, and under appropriate project and personal licences.

Electrically-stimulated efflux of [^{3}H]D-asp from rat forebrain slices was enhanced in a concentration-dependent manner by the Group I mGlu receptor agonist (S)-3,5-DHPG (0.1–3.0 μM) with statistical significance being reached at 1 and 3 μM (Fig. 1A). Higher doses of the agonist did not evoke greater responses than those seen following (S)-3,5-DHPG, 3 μM (data not shown). The EC_{50} for (S)-3,5-DHPG for this response was 1.10 μM (GraphPad, Prism software). The agonist showed no significant effects on basal efflux of label within the dose

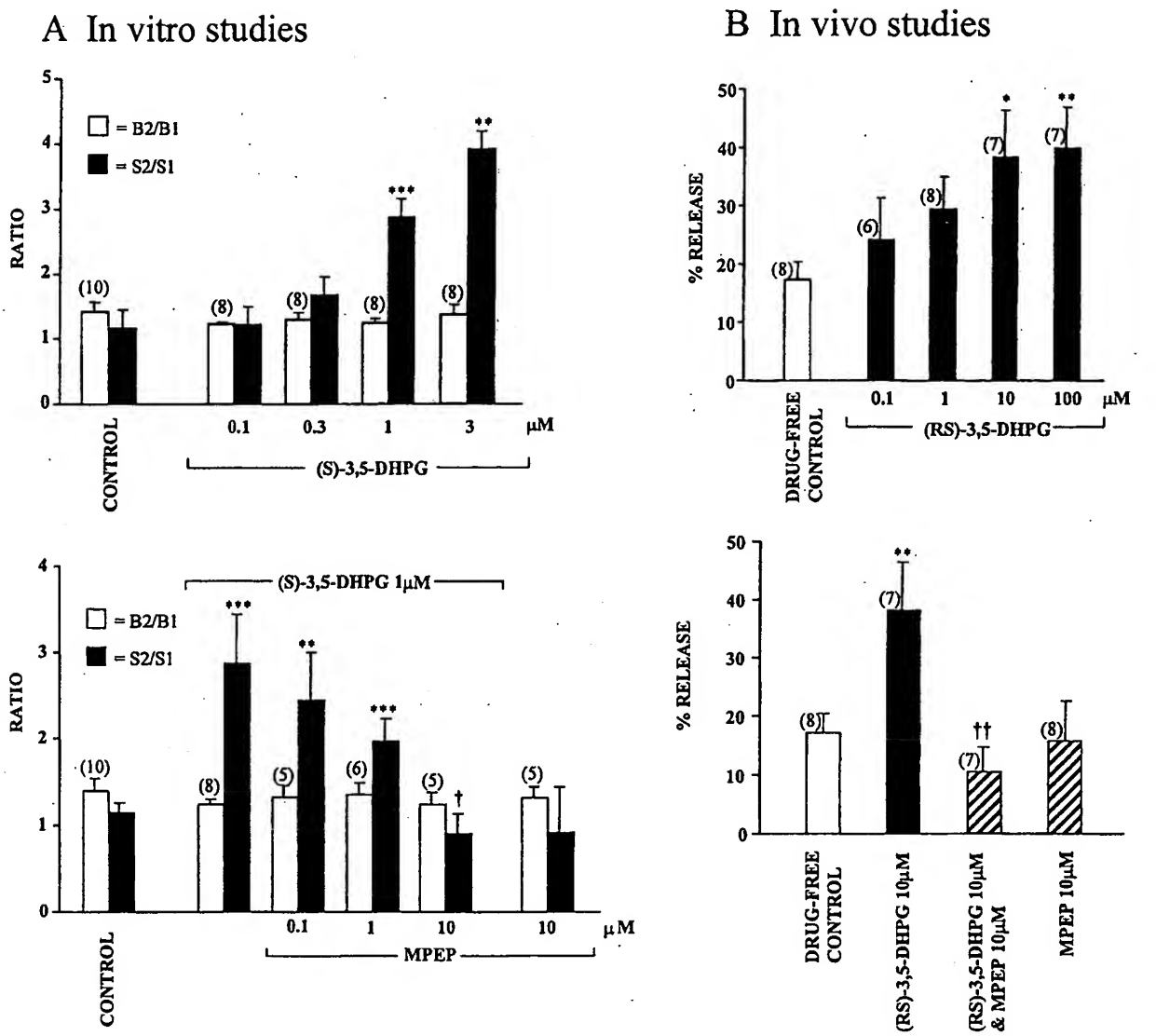

Fig. 1. Effects of the novel, selective $mGlu_5$ receptor antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) on neuronal excitatory amino acid release in vitro (A) and in vivo (B). The first two panels (A) show the influence of the Group I mGlu receptor agonist (S)-3,5-DHPG on electrically-stimulated release of [^{3}H]D-aspartate from rat forebrain slices and the concentration-dependent attenuation of this response by MPEP. In (B) the effects of (RS)-3,5-DHPG, administered by reverse dialysis, on the efflux of preloaded [^{3}H]L-glutamate from the rat corpus striatum in vivo is shown, together with the powerful inhibitory action of MPEP on these (RS)-3,5-DHPG-evoked responses. Values shown are means (±SEM) of *n* (in parenthesis) independent observations. See text for details of procedures. * = $P<0.05$, ** = $P<0.01$, *** = $P<0.005$ compared to control values; † = $P<0.05$, †† = $P<0.01$ compared to agonist alone (ANOVA followed by Student's *t*-test for independent groups). (S)-3,5-DHPG = (S)-3,5-dihydroxyphenylglycine; (RS)-3,5-DHPG = (RS)-3,5-dihydroxyphenylglycine; MPEP = 2-methyl-6-(phenylethynyl)-pyridine.

range tested (Fig. 1A). In order to test our hypothesis that presynaptic $mGlu_5$ receptors were responsible for the enhanced neurotransmitter release observed, the influence of the novel, selective $mGlu_5$ receptor antagonist MPEP (0.1–10 μM) against the responses evoked by (S)-3,5-DHPG, 1 μM was assessed. A maximal concentration of 10 μM was chosen due to the observed inhibitory effects of MPEP on NMDA receptors at higher concentrations, i.e. 100–200 μM (Gasparini et al., 1999; Attucci et al., 2001). The antagonist potently inhibited (S)-3,5-DHPG-enhanced release of electrically-stimulated [^{3}H]D-asp in a concentration-sensitive manner, with complete abolition of the response being achieved by MPEP, 10 μM (Fig. 1A). The calculated IC_{50} value for MPEP for inhibition of (S)-3,5-DHPG-evoked responses was 0.24 μM (GraphPad, Prism software). The IC_{50} value and the maximal inhibitory effect observed at 10 μM for MPEP confirm the exclusive involvement of the $mGlu_5$ receptors. Administration of MPEP alone, at the highest concentration utilised (10 μM), had no effect on the basal or electrically-stimulated release of radiolabel (Fig. 1A). To determine whether these novel

facilitatory $mGlu_5$ autoreceptors are functionally active in vivo, (RS)-3,5-DHPG (0.1–100 μM) was applied to the corpus striatum of conscious, freely moving rats, via reverse dialysis through a microdialysis monoprobe. In a manner similar to that in vitro, (RS)-3,5-DHPG evoked a concentration-dependent increase in release of [^{3}H]L-glu into the dialysis stream. When compared to drug-free control responses, significant increases in [^{3}H]L-glu release were observed following (RS)-3,5-DHPG, 10–100μM (Fig. 1B). A maximal 21.0% increase in [^{3}H]L-glu release compared to drug-free control response was attained following (RS)-3,5-DHPG, 100 μM, whilst the estimated EC_{50} concentration for (RS)-3,5-DHPG at this population of presynaptic facilitatory Group I mGlu receptors in vivo was calculated as 1.67 μM (GraphPad, Prism software). Co-administration of MPEP, 10 μM with (RS)-3,5-DHPG, 10 μM resulted in full inhibition of the (RS)-3,5-DHPG-mediated increase in [^{3}H]L-glu release (Fig. 1B). The antagonist, when applied alone at the same concentration, did not significantly influence [^{3}H]L-glu release (Fig. 1B). No marked behavioural changes were observed following intrastriatal administration of (RS)-3,5-DHPG, MPEP, or the combined administration of these ligands by reverse dialysis, with all animals remaining alert but generally inactive throughout the experiments.

The data reported here provide compelling evidence for a role of presynaptic $mGlu_5$-type glutamate receptors in the regulation of neuronal glutamate release in the mammalian central nervous system. They also provide the first demonstration of functional presynaptic $mGlu_5$ receptors in conscious, freely moving animals. The absolute inhibitory actions of MPEP against the agonist-evoked responses in both the rat forebrain in vitro and during microdialysis of the corpus striatum in vivo, suggests that activation of presynaptic $mGlu_5$ receptors alone can fully account for the positive modulatory responses observed in these areas. Consistent with this conclusion, a recent study by Sistiaga and co-workers (1998)) using knockout mice, clearly showed that nerve terminals from $mGlu_1$ receptor-deficient mice exhibit all the responses of Group I mGlu receptors observed in wild-type mice, including (RS)-3,5-DHPG-induced production of diacylglycerol (DAG) and the facilitatory influence of the agonist on neuronal glutamate release. Others have also shown that whereas presynaptic effects of (1S, 3R)-ACPD can still be observed in the hippocampal formation of $mGlu_1$ receptor-deficient mice (Aiba et al., 1994), these effects are absent in $mGlu_5$ receptor-deficient animals (Lu et al., 1997). In pharmacological studies, we have also previously demonstrated a lack of inhibitory activity of a wide range of $mGlu_1$ receptor-selective antagonists against presynaptic Group I mGlu agonist-evoked responses in the rat forebrain (Thomas et al., 2000). Additionally, in similar experiments, the selective $mGlu_5$ receptor *agonist* (RS)-2-chloro-5-hydroxyphenylglycine ((RS)-CHPG) produced a marked enhancement of neuronal excitatory amino acid release (Thomas et al., 2000). The lack of activity of MPEP alone on drug-free control responses in the present studies indicates a lack of *tonic* activation of presynaptic $mGlu_5$ autoreceptors, either in vitro or in vivo, by the presumed endogenous agonist glutamate under resting conditions. Rather, presynaptic positive modulatory glutamate autoreceptors appear to permit the maintenance of "supply on demand" for neuronal glutamate release, so ensuring a maximal postsynaptic response to particularly intense presynaptic fibre activity. At least 2 different splice variants of $mGlu_5$ receptors ($mGlu_{5a}$ and $mGlu_{5b}$) have been identified to date (Conn and Pin, 1997). The development of antagonists acting selectively at presynaptic $mGlu_5$ glutamate autoreceptor subtypes may provide new classes of drugs that could have a significant impact on the future treatment of a range of neurodegenerative disorders, including epilepsy and ischaemic brain damage, which are known to be associated with abnormally elevated levels of extracellular glutamate in the brain.

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Group I metabotropic glutamate receptors modulate glutamate and γ-aminobutyric acid release in the periaqueductal grey of rats

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Abstract

In this study, we investigated the effects of group I metabotropic glutamate (mglu) receptor ligands on glutamate and γ-aminobutyric acid (GABA) extracellular concentrations at the periaqueductal grey level by using in vivo microdialysis. An agonist of group I mglu receptors, (*S*)-3,5-dihydroxyphenylglycine [(*S*)-3,5-DHPG, 1 and 2 mM], as well as a selective agonist of $mglu_5$ receptors, (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG, 2 and 4 mM), both increased dialysate glutamate and GABA concentrations. 7-(Hydroxyimino)cyclopropa-[*b*]-chromen-1α-carboxylate ethyl ester (CPCCOEt, 1 mM), a selective $mglu_1$ receptor antagonist, and 2-methyl-6-(phenylethynyl)pyridine (MPEP, 0.5 mM), a selective $mglu_5$ receptor antagonist, perfused in combination with DHPG, antagonized the effect induced by DHPG on the extracellular glutamate and GABA concentrations. MPEP (0.5 mM), perfused in combination with CHPG, antagonized the increased glutamate and GABA extracellular levels induced by CHPG. MPEP (1 mM) decreased the extracellular concentrations of glutamate but did not modify the dialysate GABA concentrations. Moreover, as the intra-periaqueductal grey perfusion of (*RS*)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid [(*RS*)-CPP, 100 μM], a selective *N*-methyl-D-aspartate (NMDA) glutamate receptor antagonist, did not change the extracellular concentrations of glutamate, this suggests that the MPEP-induced decrease in glutamate is not a consequence of NMDA receptor blockade. These data show that group I mglu receptors in the periaqueductal grey may modulate the release of glutamate and GABA in awake, freely moving rats. In particular, $mglu_5$, but not $mglu_1$, receptors seem to be functionally active on glutamate terminals.

Keywords: mglu receptor; Amino acid neurotransmitter; Microdialysis; Periaqueductal grey

1. Introduction

Glutamate plays a critical role as excitatory neurotransmitter in the central nervous system, and its effects are mediated by activation of ionotropic and metabotropic receptors (Nakanishi et al., 1998). Metabotropic glutamate (mglu) receptors are a heterogeneous family of G-protein-coupled receptors linked to multiple second messengers and modulation of ion channel function in the nervous system (Conn and Pinn, 1997; Knöpfel et al., 1995). Their classification into three groups (groups I–III) was determined by similarities in coupling mechanisms, molecular structure, homology of sequence and pharmacology (Nakanishi, 1994). Group I receptors ($mglu_1$ and $mglu_5$, and their splice variants) are positively linked to phospholipase C and, therefore, their activation results in increased phosphoinositide turnover. Localization of $mglu_1$ and $mglu_5$ receptors on the postsynaptic terminal seems to be under the regulation of a small family of "Homer" proteins (Brakeman et al., 1997; Ciruela et al., 1999). These glutamate receptors not only modulate the function of glutamatergic neurons but also are able to change the activity of inhibitory neurons via excitatory glutamatergic inputs to these neurons. Biochemical studies in a number of preparations consistently indicate that activation of groups II and III mglu receptors leads to

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suppression of the release of excitatory and inhibitory amino acids, whereas group I enhances the release of glutamate and γ-aminobutyric acid (GABA) (Cartmell and Schoepp, 2000). A specific role for $mglu_1$ and $mglu_5$ receptors in nociceptive processing has long been demonstrated in the dorsal horn by pharmacological, immunohistochemical and in situ hybridisation (Berthele et al., 1999; Bond and Lodge, 1995; Dickenson et al., 1997; Fisher and Coderre, 1996; Jia et al., 1999). The control of spinal cord nociception is subject to supraspinal, neuronal centres, including the midbrain periaqueductal grey, the medullary nucleus raphe magnus and the adjacent medullary reticular formation (Basbaum and Fields, 1984; Duggan and Griersmith, 1979; Liebeskind et al., 1973). Since 1969, when it was demonstrated that stimulation of the periaqueductal grey produced analgesia, many studies have shown that this effect is the result of complex processes mediated by the periaqueductal grey (Reynolds, 1969). Besides glutamate and opioids, several other neurotransmitters in the periaqueductal grey participate in the control of nociception (Behbehani and Fields, 1979; Millan et al., 1987). Among these, GABA and glycine seem to play a crucial role in the processing of pain within this area (Maione et al., 1999, 2000; Moreau and Fields, 1986). In the current study, we analysed the possible participation of $mglu_1$ and $mglu_5$ glutamate receptors in the control of glutamate and GABA release in periaqueductal grey matter. In particular, we considered it interesting to explore whether periaqueductal grey functionally counteracting neurotransmissions, like glutamate and GABA, would be modulated at the same time by group I mglu subtype receptors. In our opinion, a better understanding of these processes might provide further insight into the pathophysiology of pain syndromes and possibly in changes in the functioning of the endogenous antinociceptive pathway. Previous studies in fact assessed the in vivo modification of glutamate or GABA (although not in the periaqueductal grey) induced by either stimulation or blockade of group I mglu receptors in some areas of the central nervous system (Battaglia et al., 2001; Cartmell and Schoepp, 2000; Cozzi et al., 1997, 2002; Herrero et al., 1992; Pellegrini-Giampietro et al., 1999). Although these previous findings confirmed the difficulty of determining the source of basal glutamate or GABA, they consistently found that group I mglu receptor stimulation increased both glutamate and GABA release. In particular, the group I mglu receptor-mediated increase in activity of GABAergic inputs could ultimately result in decreased excitatory neurotransmission.

Therefore, as the presence of mglu receptors in the periaqueductal grey has been shown by autoradiographic, immunostaining and pharmacological studies, and no study to date has investigated their possible role in the modulation of glutamate and GABA release at that level (Azcue et al., 1997; Catania et al., 1994; Leyva et al., 1995; Maione et al., 1998a, 2000), we evaluated this possibility in awake, freely moving rats.

2. Materials and methods

2.1. Animals

Male Wistar rats (250–300 g) were housed three per cage under controlled illumination (12:12-h light/dark cycle; light on 06.00 h) and environmental conditions (ambient temperature, 20–22 °C; humidity, 55–60%) for at least 1 week before the experiments started. Rat chow and tap water were available ad libitum. The experimental procedures were approved by the Animal Ethics Committee of the Second University of Naples. Animal care was in compliance with Italian (D.L. 116/92) and EEC (O.J. of E.C. L358/1 18/12/86) regulations on the protection of laboratory animals. All efforts were made to minimise animal suffering and to reduce the number of animals used.

2.2. Microdialysis procedure

Brain microdialysis experiments were performed in awake and freely moving rats as described previously (Biggs et al., 1992). In brief, rats were anaesthetised with chloral hydrate (400 mg/kg, i.p.) and stereotaxically implanted with concentric microdialysis probes, which were constructed, as previously described, into the ventrolateral periaqueductal grey using coordinates A: −7.5 mm from bregma, L: +0.5 mm and V: 7.7 mm below the dura (Hutson et al., 1985; Maione et al., 1998b; Paxinos and Watson, 1986). Following a postoperative recovery period of approximately 18 h, dialysis was commenced with an artificial cerebrospinal fluid (ACSF, composition in millimolar: KCl, 2.5; NaCl, 125; $MgCl_2$, 1.18; $CaCl_2$, 1.26). ACSF (pH 7.2) was perfused at a rate of 0.8 μl/min using a Harvard Apparatus infusion pump (mod. 22), and following an initial 60 min equilibration period (two discarded samples), 12 consecutive 30-min dialysate samples were collected. Rats received selective mglu receptor agonists, alone or in combination with the corresponding antagonists, directly through the dialysis probe (30-min perfusion). On completion of each experiment, rats were anaesthetised with pentobarbitone sodium and their brains were perfused-fixed via the left cardiac ventricle with heparinised paraformaldehyde saline (4%). Brains were removed 120 min following fixation, and coronal sections were cut in order to verify probe placements. Dialysates were analysed for amino acid content using a high-performance liquid chromatography (HPLC) method. The system comprised two Gilson pumps (mod. 303), a C18 reverse-phase column, a Gilson refrigerated autoinjector (mod. 231) and a Gilson fluorimetric detector (mod. 121). Dialysates were precolumn derivatised with *o*-phthaldialdehyde (10 μl dialysate + 10 μl *o*-phthaldialdehyde), and amino acid conjugates were resolved using a gradient separation. The detection limit of GABA and glutamate in 10-μl samples was about 0.5–1 and 2–3 pmol, respectively. The mobile phase consisted of two compo-

nents: (A) 50 mM sodium dihydrogen orthophosphate, pH 5.5, with 20% methanol and (B) 100% methanol. Gradient composition was determined with an Apple microcomputer installed with Gilson gradient management software, and the mobile phase flow rate was maintained at 1.0 ml/min. Data were collected by a Dell Corporation PC system 310 interfaced to the detector via a Drew data collection unit.

2.3. Immunohistochemistry

Animals were anaesthetised with sodium pentobarbitone and perfused with 4% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were removed, and transverse sections (60 μm thick) of mesencephalon were cut using a vibratome. After preincubation with normal goat serum, the sections were incubated overnight at 4 °C with either anti-mglu$_1$ or anti-mglu$_5$ polyclonal antibodies (Upstate Biotechnology, Lake Placid, NY). After two washes with phosphate buffer solution, the sections were incubated with a biotinylated secondary antibody by using the avidin–biotin peroxidase procedure

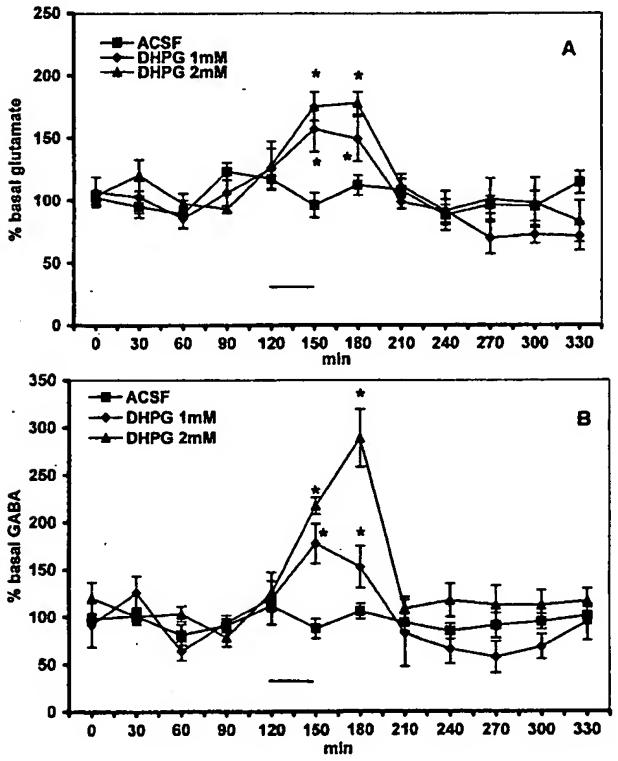

Fig. 1. Effect of artificial cerebrospinal fluid (ACSF) (A and B) or (*RS*)-3,5-DHPG (DHPG, 1 and 2 mM) on periaqueductal grey matter dialysate levels of glutamate (A) and GABA (B). The bars indicate the period of (*RS*)-3,5-DHPG perfusion. Data (five to eight rats per group) are means ± S.E.M. of amino acid extracellular concentrations expressed as percentages of the basal values. The mean basal extracellular GABA and glutamate levels (not corrected for probe recovery) were 6.1 ± 0.7 and 32 ± 5 pmol/10 μl of dialysate (means ± S.E.M.), respectively. *Significant difference versus the vehicle. *P* values <0.05 were considered statistically significant.

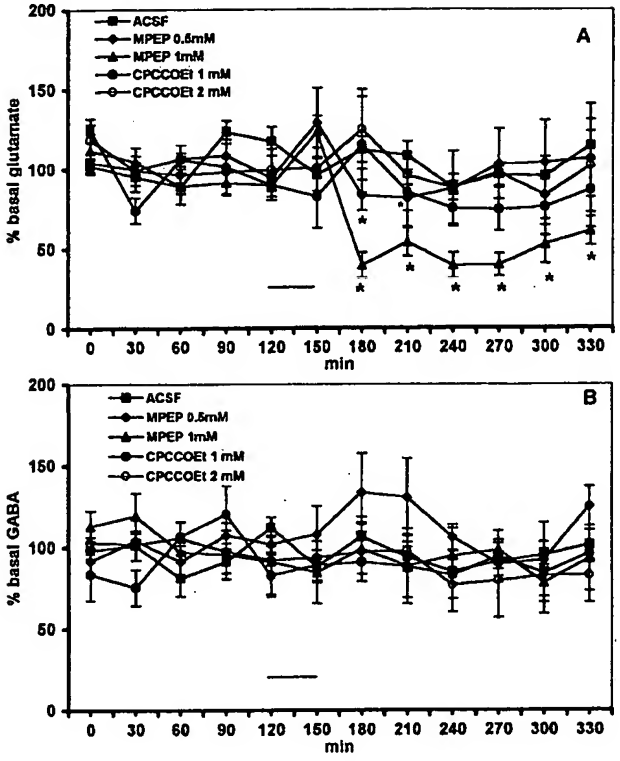

Fig. 2. Effect of artificial cerebrospinal fluid (ACSF) (A and B) CPCCOEt (1 and 2 mM) or MPEP (0.5 and 1 mM) on periaqueductal grey matter dialysate levels of glutamate (A) and GABA (B). The bars indicate the period of MPEP or CPCCOEt perfusion. Data (six to eight rats per group) are means ± S.E.M. of amino acid extracellular concentrations expressed as percentages of the basal values. The mean basal extracellular GABA and glutamate levels (not corrected for probe recovery) were 5.9 ± 0.7 and 33 ± 6 pmol/10 μl of dialysate (means ± S.E.M.), respectively. *Significant difference versus the vehicle. *P* values <0.05 were considered statistically significant.

(Vector Laboratories, Burlingame, CA) and diaminobenzidine as chromogen. The sections were then treated with 1% osmium, stained with uranyl acetate, dehydrated in graded solutions of acetone and embedded in araldite. Ultrathin sections were cut and analysed using a Philips CM10 electron microscope. The negative controls were made by omitting the primary antibodies and using nonimmune antisera, which resulted in complete absence of staining.

2.4. Drugs

The following drugs were used: (*S*)-3,5-dihydroxyphenylglycine [(*S*)-3,5-DHPG], (*RS*)-2-chloro-5-hydroxyphenylglycine (CHPG), 7-(hydroxyimino)cyclopropa-[*b*]-chromen-1α-carboxylate ethyl ester (CPCCOEt), 2-methyl-6-(phenylethynyl)pyridine (MPEP), tetrodotoxin and (*RS*)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid [(*RS*)-CPP] (Tocris Cookson, Bristol, UK). CPCCOEt was dissolved in 10% dimethylsulphoxide in ACSF (pH 7.2). All the other drugs were dissolved in ACSF with final pH of 7.2.

2.5. Statistics

Statistical analysis of the data was performed by analysis of variance (ANOVA) followed by the Student–Newman–Keuls multiple comparisons test. Differences were considered significant at $P<0.05$.

3. Results

The mean basal extracellular GABA, glutamate and glutamine levels in the periaqueductal grey (not corrected for probe recovery of 28 ± 6%, 32 ± 4% and 30 ± 7% for GABA, glutamate and glutamine, respectively) were 6.4 ± 0.4, 28 ± 5 and 422 ± 18 pmol/10 μl of dialysate (means ± S.E.M.), respectively. These values are in accordance with those obtained in our previous studies and in other laboratories (Renno et al., 1992; Maione et al., 1999, 2000). Each animal was used once only, and the reported basal values of glutamate, GABA and glutamine are the mean concentrations obtained from all experiments. Intra-periaqueductal grey perfusion of tetrodotoxin (1 μM) decreased the extracellular levels of glutamate and GABA (48 ± 6% and 53 ± 7% for glutamate and GABA, respectively), but not glutamine (data not shown). Intra-periaqueductal grey perfusion of (*S*)-3,5-DHPG (1 and 2 mM), a selective agonist of group I mglu receptors, increased the dialysate glutamate (156 ± 18% and 177 ± 10%, respectively) and GABA (177 ± 23% and 288 ± 20%, respectively) concentrations (Fig. 1). CPCCOEt (2 and 4 mM), as well as its vehicle (dimethylsulphoxide, 10% in ACSF), did not modify per se dialysate glutamate and GABA levels (Fig. 2). MPEP (0.5–1 mM) decreased, in a concentration-dependent manner, the dialysate glutamate concentrations (– 58 ± 7%) without affecting GABA concentrations (Fig. 2). Intra-periaqueductal grey perfusion of (*RS*)-CPP (100 μM), a selective *N*-methyl-D-aspartate (NMDA) receptor antagonist, did not modify the extracellular levels of glutamate (data not shown). CPCCOEt (1 mM), a selective $mglu_1$ receptor antagonist, and MPEP (0.5 mM), a selective $mglu_5$ receptor antagonist, perfused in combination with (*S*)-3,5-

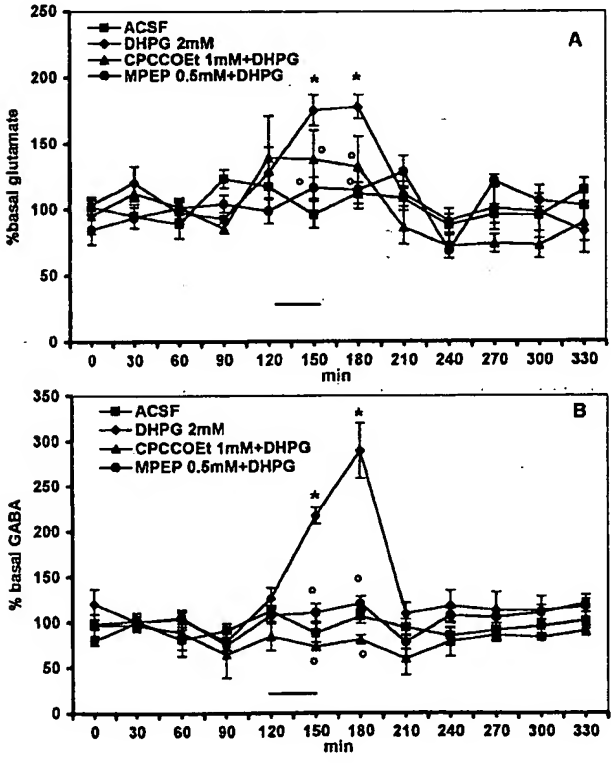

Fig. 3. Effects of artificial cerebrospinal fluid (ACSF) (A and B) (*RS*)-3,5-DHPG (DHPG, 2 mM), alone or in combination with CPCCOEt (1 mM) or MPEP (0.5 mM), on periaqueductal grey matter dialysate levels of glutamate (A) and GABA (B). The bars indicate the period of drug perfusion. Data (five to eight rats per group) are means ± S.E.M. of amino acid extracellular concentrations expressed as percentages of the basal values. The mean basal extracellular GABA and glutamate levels (not corrected for probe recovery) were 6.9 ± 0.8 and 29 ± 6 pmol/10 μl of dialysate (means ± S.E.M.), respectively. *Significant difference versus the vehicle. °Significant difference versus DHPG 2 mM. *P* values <0.05 were considered statistically significant.

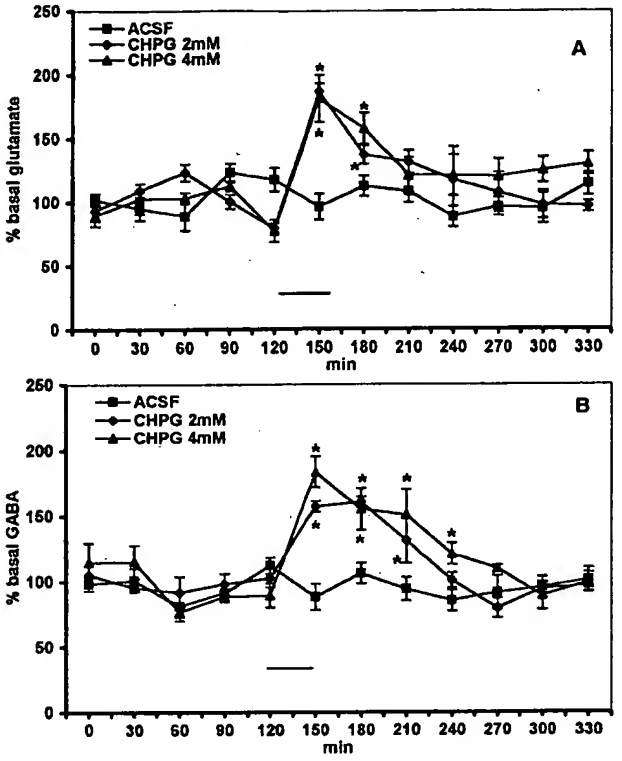

Fig. 4. Effect of artificial cerebrospinal fluid (ACSF) (A and B) or CHPG (2 and 4 mM) on periaqueductal grey matter dialysate levels of glutamate (A) and GABA (B). The bars indicate the period of CHPG perfusion. Data (five to eight rats per group) are means ± S.E.M. of amino acid extracellular concentrations expressed as percentages of the basal values. The mean basal extracellular GABA and glutamate levels (not corrected for probe recovery) were 6.6 ± 0.8 and 26 ± 7 pmol/10 μl of dialysate (means ± S.E.M.), respectively. *Significant difference versus the vehicle. *P* values <0.05 were considered statistically significant.

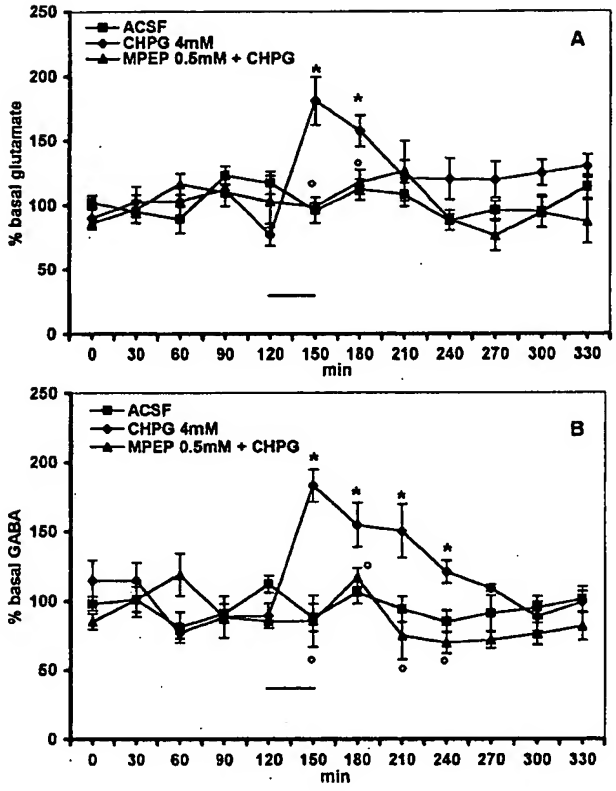

Fig. 5. Effects of artificial cerebrospinal fluid (ACSF) (A and B) or CHPG (4 mM), alone or in combination with MPEP (0.5 mM), on periaqueductal matter dialysate levels of glutamate (A) and GABA (B). The bars indicate the period of drug perfusion. Data (five to eight rats per group) are means ± S.E.M. of amino acid extracellular concentrations expressed as percentages of the basal values. The mean basal extracellular GABA and glutamate levels (not corrected for probe recovery) were 6.9 ± 0.5 and 30 ± 40 pmol/10 μl of dialysate (means ± S.E.M.), respectively. *Significant difference versus the vehicle. °Significant difference versus CHPG 4 mM. P values <0.05 were considered statistically significant.

DHPG, antagonized the (*S*)-3,5-DHPG-induced increase in extracellular concentrations of glutamate and GABA (Fig. 3). CHPG (2 and 4 mM), a selective agonist of $mglu_5$ receptors, increased the dialysate glutamate (186 ± 5% and 180 ± 18%, respectively) and GABA (160 ± 5% and 183 ± 11%, respectively) concentrations (Fig. 4). With (*S*)-3,5-DHPG or CHPG, the changes in amino acid extracellular values were greatest for GABA. MPEP (0.5 mM), a selective $mglu_5$ receptor antagonist, perfused in combination with CHPG (4 mM), antagonized the CHPG-induced increase in glutamate and GABA extracellular concentrations (Fig. 5). The extracellular concentrations of glutamine never changed following treatment with tetrodotoxin (1 μM), (*S*)-3,5-DHPG, CHPG, CPCCOEt and MPEP (data not shown). At the ultrastructural level, immunostaining for $mglu_1$ and $mglu_5$ receptors was mainly localized in the dendrites of periaqueductal grey neurons (Fig. 6); however, some perikarya showed weak $mglu_5$ positivity. Many of the $mglu_1$- and $mglu_5$-positive dendrites received synapses containing round vesicles.

4. Discussion

There is an evidence that ionotropic and metabotropic glutamate receptors are expressed on both neural synaptic and astrocytic processes (Gallo and Ghiani, 2000). Although glutamate released from neurons can activate glutamate receptors on glia to cause changes such as (i) transmitter uptake into glial cells, (ii) modulation of K^+ conductances and (iii) release of neuroactive substances from glia that can modulate synaptic transmission (Vernadakis, 1996; Araque et al., 1999), there is no way in this study to distinguish between glial or neural dialysate amino acids either before or after mglu receptor stimulation. Nevertheless, the changes in periaqueductal grey glutamate or GABA levels may deeply affect nociceptive perception, as this midbrain area is part of the endogenous antinociceptive system (Gebhart et al., 1984). In this study, the possible synaptic nature of periaqueductal grey dialysate glutamate and GABA seems confirmed in part

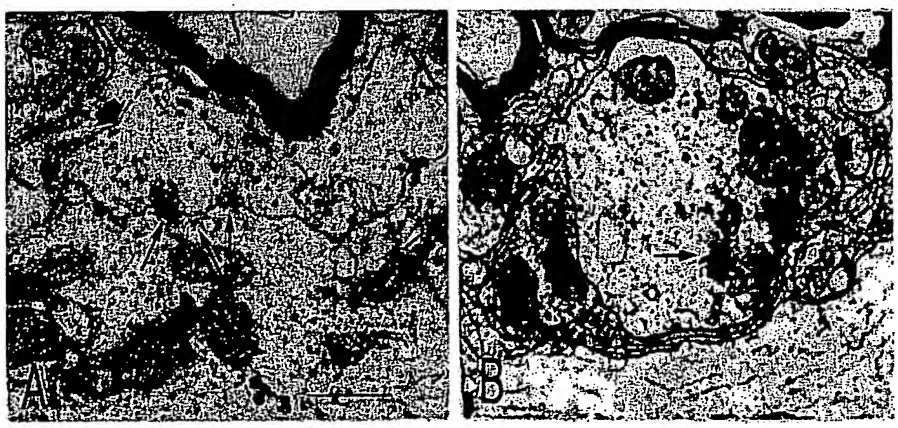

Fig. 6. Photomicrograph of $mglu_1$ (A)- and $mglu_5$ (B)-positive dendrites in the periaqueductal grey. The arrows indicate reaction product. The arrowhead indicates the axon terminal. Bar: 1 μm.

by the fact that tetrodotoxin almost halved their extracellular concentrations. This finding suggests that almost 40% of extracellular GABA or glutamate in the periaqueductal grey may function as neurotransmitter. Moreover, this study provides pharmacological evidence in vivo that intra-periaqueductal grey perfusion of (*S*)-3,5-DHPG, an agonist of group I mglu receptors, and CHPG, a selective agonist of $mglu_5$ receptors, leads to an increase in glutamate and GABA extracellular concentrations. Contrary to what was observed with glutamate and GABA, the extracellular concentration of glutamine never changed with either (*S*)-3,5-DHPG or CHPG. This confirms that glutamine is not synaptically released and is mainly important in amino acid metabolic pathways (Maione et al., 2000). With (*S*)-3,5-DHPG or CHPG treatment, the change in amino acid extracellular levels was greatest for GABA. The postsynaptic nature of group I mglu receptors, as well as the huge population of GABAergic interneurons in the periaqueductal grey (possibly expressed at the somato-dendritic level), may underlie this difference (Lujan et al., 1996; Moreau and Fields, 1986). The selective $mglu_1$ receptor antagonist CPCCOEt was used in combination with (*S*)-3,5-DHPG in order to discriminate which mglu subtype receptor was involved. This antagonist was found to antagonize the effect of (*S*)-3,5-DHPG on both glutamate and GABA extracellular values. Since CHPG also increased extracellular concentrations of glutamate and GABA, the involvement of $mglu_5$ receptors was suggested. Compared to (*S*)-3,5-DHPG, CHPG induced a smaller increase in glutamate and GABA levels, and this may be the consequence of its lower potency (Doherty et al., 1997). Also, CHPG did not generate a concentration-dependent change in the extracellular levels of these amino acids, presumably because very similar concentrations (2 and 4 mM) of this drug were perfused. Intra-periaqueductal grey treatment with MPEP, a selective $mglu_5$ receptor antagonist, perfused in combination with CHPG, further confirmed the involvement of $mglu_5$ receptors in the modulation of glutamate and GABA release. Moreover, $mglu_5$ receptors seem to exert a tonic control of glutamate release at the periaqueductal grey level as their blockade with MPEP reduced per se the extracellular concentrations of this amino acid. The possible involvement of NMDA receptors in this effect was ruled out by the fact that (*RS*)-CPP, a selective NMDA receptor antagonist, did not decrease the glutamate extracellular level in the periaqueductal grey. The blockade of $mglu_1$ receptors with CPCCOEt had no effect either on glutamate or GABA extracellular level. In agreement with previous studies (Cozzi et al., 2002; Lorrain et al., 2002; Maione et al., 1998b; Neugebauer et al., 1999; Pintor et al., 2000), the concentration of mglu receptor ligands used in this study was higher than their in vitro EC/IC_{50} (millimolar versus micromolar) values. The fact that micromolar concentrations were ineffective may be possibly due to (i) the relatively low probe recovery (20–30%), (ii) their uptake and metabolism by glial and neural cells or (iii) drug diffusion from probe site. We suppose that due to these reasons, the actual drug concentrations reaching cerebral tissue resemble those used in the in vitro studies (Schoepp et al., 1999). Since MPEP decreased extracellular concentrations of glutamate in the periaqueductal grey, a role of presynaptic $mglu_5$ receptors may be suggested in this response. In agreement with this concept, intracerebral microdialysis revealed a presynaptic mglu receptor-mediated enhancement of [^{3}H]L-glutamate and endogenous transmitter release from the rat striatum and nucleus tractus solitarius (Jones et al., 1998a; Patel and Croucher, 1998). This is in agreement with a recent study by Thomas et al. (2000, 2001), showing that presynaptically located $mglu_5$ receptors positively modulate neuronal glutamate release in the rat forebrain in the presence of selective agonists. We recently showed that microinjections of MPEP into the periaqueductal grey of rat reduced the latency of the nociceptive reaction in the plantar test (Palazzo et al., 2001). This suggests that glutamate may physiologically modulate the endogenous antinociceptive pathway since decreased $mglu_5$ receptor activation may generate a persistent hyperalgesic effect. However, it also has been suggested that $mglu_5$ receptors serve as autoreceptors to inhibit synaptic transmission in the hippocampal area CA1 (Manzoni and Bockaert, 1995). Moreover, $mglu_1$ and $mglu_5$ receptors may exert opposite effects in primate spinothalamic tract neurons since (*S*)-3,5-DHPG potentiated and CHPG decreased the responses to brief innocuous and noxious mechanical stimulation (Neugebauer et al., 1999). In agreement with a previous neuroanatomical study (Azcue et al., 1997), we showed in this study that $mglu_5$, as well as $mglu_1$, immunoreactivity was located in cell bodies and dendritic processes. Axon terminals making synaptic contact with both $mglu_1$- and $mglu_5$-immunoreactive dendrites contained round clear vesicles. Therefore, in the light of these morphological findings, we can rule out the possibility that dialysate glutamate levels were modulated presynaptically by $mglu_5$ receptors.

In addition, this study suggests that group I mglu receptors regulate GABA release in the periaqueductal grey of awake, freely moving rat. This is shown by the finding that CPCCOEt and MPEP, selective antagonists of $mglu_1$ and $mglu_5$ receptors, respectively, antagonized the (*S*)-3,5-DHPG- and CHPG-induced increase in dialysate GABA levels. Previous studies showed either an augmentation of KCl-evoked GABA release or an increased release of this amino acid by selective mglu agonists in slices of rat striatum (Wang and Johnson, 1995; Wang et al., 1996). Also, 1*S*,3*R*-ACPD and the selective group I mglu receptor agonists (*S*)-3,5-DHPG and quisqualate increased KCl-evoked [^{14}C]GABA release in superfused slices of rat nucleus tractus solitarius or potentiated increases in [^{14}C]GABA release induced by NMDA in

striatal slices (Jones et al., 1998a,b; Hanania and Johnson, 1999). The role played by $mglu_1$ and $mglu_5$ receptors in the periaqueductal grey on the physiology of GABA release is far from established in awake, freely moving rat. There is evidence that microinjections of selective GABA receptor antagonists in the periaqueductal grey induce analgesia by decreasing the tonic GABAergic function (Moreau and Fields, 1986). In this study, we reported that group I mglu receptors may positively modulate the release of GABA, which could be expected to generate hyperalgesia by inhibiting the antinociceptive pathway. However, this is in contrast with our previous observation that both acute and persistent nociceptive behaviours were decreased by intra-periaqueductal grey microinjection of group I mglu receptor agonists (Maione et al., 1998a, 2000). It is possible that the variations in the extracellular levels of these amino acids may be a result of the complex network activity in the periaqueductal grey in vivo. Under our conditions, generalised stimulation of group I postsynaptic mglu receptors in the periaqueductal grey may cause sustained activation of the output antinociceptive pathways. This may prevail over the other modulatory effects in the periaqueductal grey (i.e. the positive modulation of GABA release) and possibly mask the fine-tuning. The precise antinociceptive mechanisms induced by the selective $mglu_{1/5}$ agonists in the periaqueductal grey, in spite of the fact that GABA levels were increased, are unclear, and further investigation is needed to throw new light on the relationship between periaqueductal grey-induced analgesia and GABA and mglu receptors. However, it is worth noting that mglu receptor activation might either down-regulate or up-regulate inhibitory postsynaptic currents in the nucleus of the tractus solitarius and spinal cord in the rat (Glaum and Miller, 1993). Furthermore, activation of group I mglu receptors was found to depress $GABA_A$-mediated inhibitory postsynaptic currents (IPSCs) in slices of rat midbrain dopaminergic neurons (Bonci et al., 1997). The fact that group I mglu receptors modulate the release of excitatory and inhibitory neurotransmitters in the periaqueductal grey in the same direction may be suggestive of the physiological fine-tuning of these two counteracting neurotransmissions. As in other brain areas like the pallidum or substantia nigra (Hanson and Smith, 1999; Hubert et al., 2001), it is possible that group I mglu receptors facilitate the release of GABA at extra synaptic sites or symmetric synapses in the periaqueductal grey. This mglu receptor-mediated GABA release might be physiologically relevant to prevent excessive glutamate accumulation and, therefore, limit possible excitotoxic effects of high concentrations of glutamate.

In conclusion, this study provides evidence that $mglu_1$ and $mglu_5$ receptors may control the release of glutamate and GABA within the periaqueductal grey matter. In particular, $mglu_5$, but not $mglu_1$, receptors seem to be functionally active on postsynaptic terminals in this midbrain area, where they tonically modulate the endogenous antinociceptive pathway.

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LY354740: a Metabotropic Glutamate Receptor Agonist which Ameliorates Symptoms of Nicotine Withdrawal in Rats

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Summary—LY354740 is a conformationally constrained analog of glutamate with high selectivity and nanomolar agonist activity at Group II metabotropic glutamate receptors (mGluRs). This orally active compound is a new drug candidate which is being developed for the treatment of anxiety. In this study, LY354740 was investigated in a model of nicotine withdrawal using the acoustic startle reflex (sensorimotor reactivity) in rats. Nicotine (6 mg/kg/day) was administered for 12 days subcutaneously by osmotic minipumps. After 12 days the pumps were removed and the animals were allowed to go through spontaneous withdrawal. Cessation of chronic nicotine exposure led to increased startle responding for 4 days following withdrawal. Treatment with LY354740 (0.0001–0.1 mg/kg, i.p.; 0.03–3 mg/kg, oral) produced a dose-dependent attenuation of the enhanced auditory startle responding following withdrawal of nicotine with intraperitoneal and oral ED_{50} values of 0.003 mg/kg and 0.7 mg/kg, respectively. These effects were stereoselective since the (−)-enantiomer of LY354740, LY366563, was without effect in this model. LY354740 produced no changes in the sensorimotor reactivity of rats not exposed to nicotine at oral doses up to 10 mg/kg. These data support the functional role of mGluR agonists in nicotine withdrawal and indicate that LY354740 may be efficacious in reducing the symptoms associated with nicotine withdrawal during smoking cessation in humans.

Keywords—Metabotropic glutamate receptors (mGluRs), nicotine, withdrawal, sensorimotor reactivity, startle.

The excitatory neurotransmitter L-glutamate has been shown to activate ligand-gated cationic channels termed *N*-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) and kainate (KA) receptors (ionotropic glutamate receptors; iGluRs) (Johnson and Ascher, 1987; Honore *et al.*, 1988; Lodge and Collingridge, 1991) and to regulate ion channels and enzymes producing second messengers via specific receptors coupled to G-proteins (Baskys, 1994). In contrast to ion channel-linked glutamate receptors (ionotropic) which facilitate fast synaptic transmission, metabotropic glutamate receptors (mGluRs) represent a heterogeneous family of receptor proteins that modulate synaptic function through coupling to multiple second messenger systems (Pin and Duvoisin, 1995). Metabotropic glutamate receptors are currently classified into three groups based on sequence homology, second messenger coupling and similar agonist pharmacology. Group I mGluRs include $mGluR_1$ and $mGluR_5$, which are coupled to phosphoinositide (PI) hydrolysis when expressed in non-neuronal cells. Group II mGluRs, which include $mGluR_2$ and $mGluR_3$, are negatively coupled to cyclic adenosine 3′,5′-monophosphate (cAMP) formation. Group III mGluRs are the most heterogeneous subgroup of mGluRs, and include $mGluR_4$, $mGluR_6$, $mGluR_7$ and $mGluR_8$. Group III mGluRs are also negatively coupled to cAMP formation when expressed in non-neuronal cells (Nakanishi, 1992; Schoepp, 1994; Pin and Duvoisin, 1995). The characterization of mGluRs in the central nervous system (CNS) represents a new area of therapeutic opportunity.

LY354740 [(+)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate monohydrate] (see Fig. 1) is a structural analog of glutamate which is highly selective for Group II metabotropic glutamate receptors. LY354740 has been shown to have no agonist or antagonist activity at Group I or Group III receptors up to 100 μM (Monn *et al.*, 1997; Schoepp *et al.*, 1997). Furthermore, LY354740 (up to

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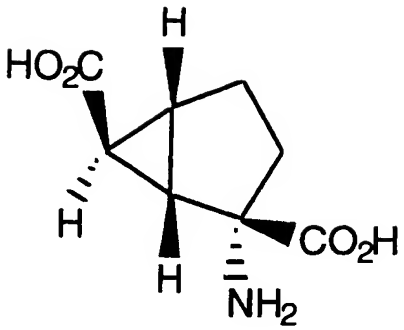

Fig. 1. Chemical structure of LY354740.

100 μM) does not displace radioligand binding at NMDA, AMPA or KA receptors (Monn *et al.*, 1997). The mGluR agonist effects of LY354740 are highly stereoselective, since LY366583, the (−) isomer of (+) LY354740, is >5000 times less active than LY354740, as shown by their EC_{50} values for decreasing forskolin-stimulated cAMP formation in rat brain slices (Monn *et al.*, 1997). LY354740, but not LY366583, has been shown to produce anxiolytic activity in the potentiated startle and elevated plus maze models of anxiety (Helton *et al.*, 1997; Monn *et al.*, 1997). As a result of these experimental findings, LY354740 is being developed for clinical investigation in the treatment of anxiety-related disorders.

Chronic administration of nicotine results in tolerance and dependence in both humans (Schachter, 1979; Shiffman and Phil, 1979; Henningfield *et al.*, 1995) and rodents (Clarke, 1987; Helton *et al.*, 1993). Cessation of chronic nicotine exposure in humans results in a number of withdrawal symptoms which include anxiety and irritability (Hughes and Hatsukami, 1992; Henningfield *et al.*, 1995). In rodents, however, signs of overt physical or behavioral withdrawal from chronic nicotine are limited. As such, there have been few robust, well accepted models for evaluating nicotine withdrawal in rodents (Emmett-Oglesby *et al.*, 1990). We have previously shown that cessation of chronic nicotine exposure in rats results in a robust increase in startle responding during the first 5 days following withdrawal, and re-administration of nicotine (0.4 mg/kg i.p.) greatly attentuates the enhanced startle response subsequent to nicotine withdrawal (Helton *et al.*, 1993). This enhanced sensorimotor responsiveness may reflect one or more of the withdrawal symptoms reported in man, and may provide a sensitive model for examining pharmacological intervention following the cessation of chronic nicotine exposure.

Metabotropic glutamate receptors can modulate both excitatory and inhibitory neuronal transmission by pre- and post-synaptic mechanisms. The role of glutamate in nicotine withdrawal is supported by an increasing number of studies reporting changes in glutamatergic function following both acute and chronic nicotine administration and/or withdrawal. For example, there have been reported interactions between the nicotinic cholinergic system and the glutamatergic system (Aizenman *et al.*, 1991; Vidal, 1994; Zhang *et al.*, 1994). Acute nicotine administration has been shown to enhance the release of glutamate through activation of nicotinic receptors located on presynaptic terminals and to facilitate evoked glutamate synaptic transmission (McGehee *et al.*, 1995; Gray *et al.*, 1996). However, the pharmacological characterization and understanding of the function of mGluRs in nicotine withdrawal have been limited due to a lack of potent, systemically active and selective pharmacological probes. In this study, the effects of the mGluR agonist, LY354740, were examined in rats undergoing withdrawal from chronic nicotine exposure. The effects of LY354740 were also compared to LY366583, which is the inactive (−) isomer of LY354740.

MATERIALS AND METHODS

Animals

Male Long–Evans rats (180–400 g) were obtained from Harlan Sprague–Dawley (Cumberland, IN, U.S.A.). All animals were acclimated for at least 3 days before testing. Animals were housed at 23 ± 2°C (relative humidity 30–70%) and given Purina Certified Rodent Chow and water *ad libitum*. The photoperiod was 12 hr light–12 hr dark, with dark onset at approximately 1800 hr. All experiments were performed in accordance with Eli Lilly and Company animal care and use policies.

Compounds

LY354740 monohydrate [(+)-2-aminobicyclo[3.1.0]-hexane-2,6-dicarboxylate monohydrate] and LY366563 [(−)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylate monohydrate] were synthesized as described by Monn *et al.* (1997). LY354740 and LY366563 were dissolved in a vehicle of purified water and neutralized with 5 N sodium hydroxide to a pH of approximately 7–8. Nicotine ditartrate (Research Biochemicals Inc., Natick, MA, U.S.A.) was dissolved in 0.9% saline. Food was removed at least 1 hr before testing.

Sensorimotor reactivity and nicotine withdrawal

For chronic nicotine administration, animals were anesthetized with isoflurane and Alzet osmotic mini-pumps (Model 2ML2; Alza Corporation, Vacaville, CA, U.S.A.) were implanted subcutaneously. Pumps were filled with nicotine ditartrate (6 mg/kg base per day) or vehicle (saline). Twelve days following implantation of pumps, rats were anesthetized with isoflurane and the pumps were removed. The auditory startle response (peak amplitude, V_{max}) of individual rats was recorded using San Diego Instruments startle chambers (San Diego, CA, U.S.A.). Startle sessions consisted of a 5-min adaptation period at a background noise level of 70 ± 2 dBA immediately followed by 25 presentations of auditory

stimuli (120 ± 3 dBA noise, 50 msec duration) presented at 8-sec intervals. Peak startle amplitudes were then averaged for all 25 presentations of stimuli for each session. All data are presented as overall session means. Baseline startle responding was evaluated prior to pump removal on day 12. Following chronic nicotine administration and removal of pumps, auditory startle responding was evaluated daily for 4 days at 24-hr intervals. Assessment began 24 hr after pump removal. LY354740 (0, 0.0001, 0.001, 0.01 or 0.1 mg/kg, i.p.; 0, 0.03, 0.3, 3 mg/kg, oral) was administered daily over the 4-day assessment period. Intraperitoneal LY354740 administration occurred 20 min prior to startle testing. Oral administration occurred 60 min prior to startle testing. The acute effects of LY354740 on startle responding was assessed in a separate experiment. In that experiment, startle responding was measured 60 min following an oral dose of 0, 1, 3 or 10 mg/kg LY354740. Startle data were analyzed using a one-way analysis of variance for each test day. When significant treatment effects were obtained, *post hoc* comparisons were made using a Tukey's studentized range (HSD) test. All statistical comparisons for nicotine withdrawal were made to the respective nicotine control group. In all analyses, two-tailed statistical tests were used and the level of significance was set at $p \leq 0.05$.

RESULTS

Sensorimotor reactivity—acute auditory startle

LY354740 had no significant effect on auditory startle responding in rats at oral doses up to 10 mg/kg (Table 1).

Sensorimotor reactivity and nicotine withdrawal

Intraperitoneal. Auditory startle responding was significantly increased (Tukey's HSD, $p \leq 0.05$) from 2 to 4 days following cessation of chronic nicotine exposure when compared to the response in control rats receiving vehicle (Fig. 2). Pretreatment with LY354740 (i.p.) produced an attenuation of the withdrawal-induced increase in startle responding with a significant attenuation at 0.001, 0.01 and 0.1 mg/kg on withdrawal days 2, 3 and 4 (Tukey's HSD, $p \leq 0.05$) when compared to nicotine controls (Fig. 2) ($ED_{50} = 0.003$ mg/kg LY354740; based on withdrawal day 2). The inactive (−) isomer of (+)LY354740, LY366583, did not attenuate the withdrawal-induced increase in startle responding at a dose of 0.1 mg/kg (i.p.) (Fig. 3).

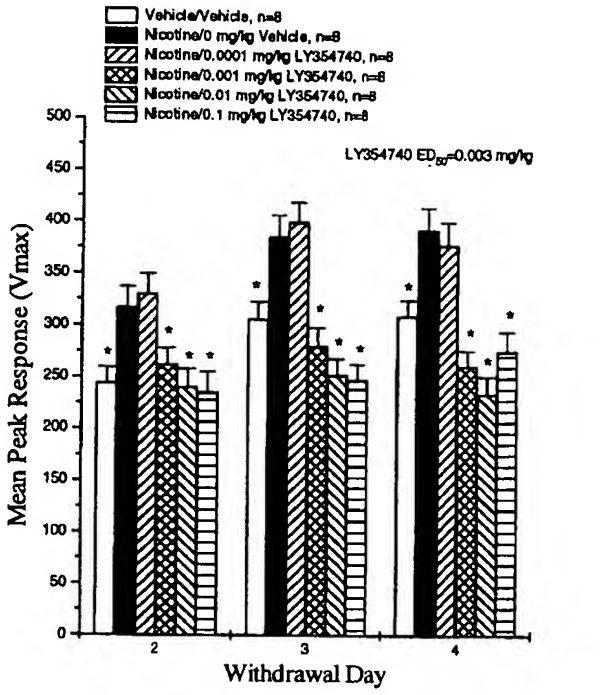

Fig. 2. Mean (±SE) peak startle amplitude (V_{max}). Auditory startle responding was evaluated on withdrawal days 2–4 at 24-hr intervals following removal of nicotine on day 12 (6 mg/kg/day, s.c.). Baseline startle responding was evaluated 24 hr before pump removal on treatment day 11 (data not shown). Vehicle or LY354740 (0, 0.0001, 0.001, 0.01 or 0.1 mg/kg, i.p.) was administered daily 20 min before startle evaluation. Vehicle represents rats administered saline for 12 days and then injected with water prior to startle assessment. Nicotine represents rats administered nicotine for 12 days and then injected with water or various doses of LY354740. *Significantly different from nicotine control, $p \leq 0.05$.

Table 1. Effect of orally administered LY354740 on sensorimotor reactivity as evaluated using auditory startle responding in Long–Evans rats

Compound	Dose (mg/kg, oral)	Startle response (μV)
Vehicle		251 ± 16
LY354740	1	212 ± 17
	3	221 ± 15
	10	300 ± 13

LY354740 was administered by oral gavage 60 min before testing. Values represent the mean (±SE) peak response (maximum startle amplitude) averaged over 25 trials. $n = 10$ rats/treatment group.

Oral. As shown in Fig. 4, LY354740 was administered orally, then tested in animals which had received either nicotine or saline vehicle by pump. In the baseline group (1 day prior to pump removal) there was no difference in startle response between animals receiving nicotine or the saline vehicle. LY354740 (0.03, 0.3 or 3 mg/kg oral, 60-min pretreatment) had no effect on baseline startle in the nicotine-treated animals. Auditory startle responding was significantly increased in nicotine-treated (pumps removed to induce withdrawal) animals (Tukey's HSD, $p \leq 0.05$) at withdrawal days 1, 2 and 3 when compared to the response in control rats (saline vehicle pumps removed) (Fig. 4). Pretreatment (60 min) with LY354740 (oral) during the withdrawal phase of the experiment produced an attenuation of the withdrawal-induced increase in startle responding, which was significant at 3 mg/kg on withdrawal days 1, 2 and 3 (Tukey's HSD,

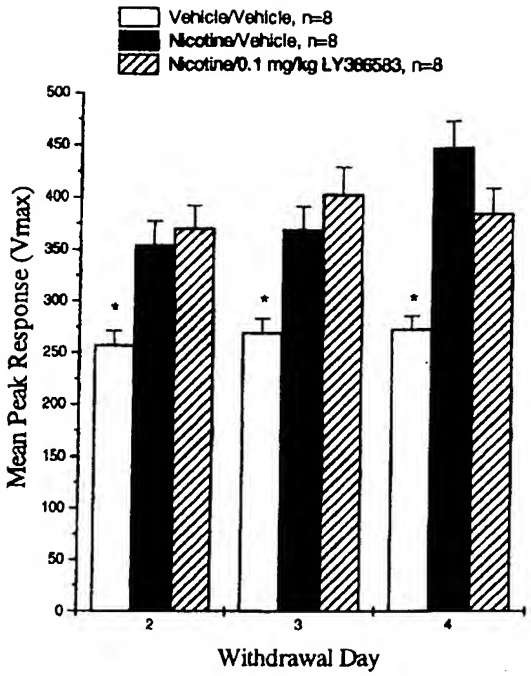

Fig. 3. Mean (±SE) peak startle amplitude (V_{max}). Auditory startle responding was evaluated on withdrawal days 2–4 at 24-hr intervals following removal of nicotine on day 12 (6 mg/kg/day, s.c.). Baseline startle responding was evaluated 24 hr before pump removal on treatment day 11 (data not shown). Vehicle or the inactive isomer (−)LY366583 (0 or 0.1 mg/kg, i.p.) was administered daily 20 min before startle evaluation. Vehicle represents rats administered saline for 12 days and then injected with water during the withdrawal period. Nicotine represents rats administered nicotine for 12 days and then injected with water or various doses of LY366583 during withdrawal. *Significantly different from nicotine control $p \leq 0.05$.

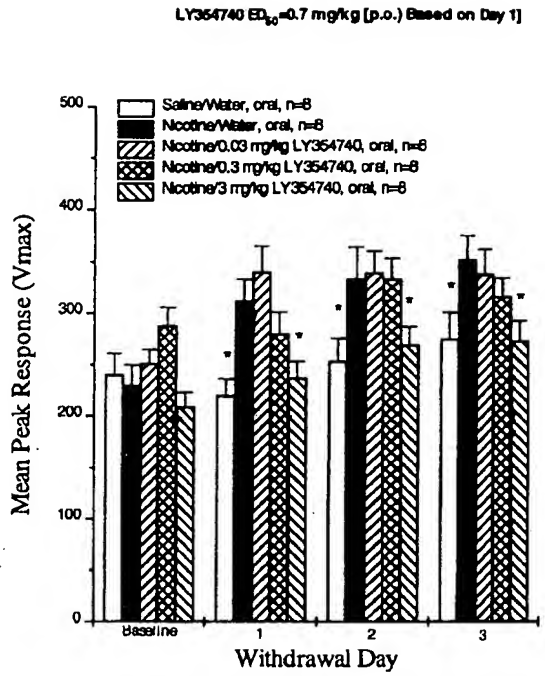

Fig. 4. Mean (±SE) peak startle amplitude (V_{max}). Auditory startle responding was evaluated on withdrawal days 1–3 at 24-hr intervals following removal of nicotine on day 12 (6 mg/kg/day, s.c.). Baseline startle responding was evaluated before pump removal on treatment day 12. Water or LY354740 (0.03, 0.3, 3 mg/kg, oral) was administered daily 60 min before startle evaluation during the withdrawal period. Saline/water represents rats administered vehicle for 12 days and then injected with water. Nicotine/water or LY354740 represents rats administered nicotine for 12 days and then injected with water or various doses of LY354740 during the withdrawal period. *Significantly different from nicotine $p \leq 0.05$.

$p \leq 0.05$) when compared to nicotine controls (Fig. 4) (ED_{50} = 0.7 mg/kg LY354740; based on withdrawal day 2).

DISCUSSION

The effects of LY354740 on the increased auditory startle responding seen in rats undergoing withdrawal from chronic administration of nicotine were evaluated to determine the potential use of LY354740 as an aid in nicotine withdrawal and/or smoking cessation. Increases in sensorimotor reactivity observed following withdrawal of nicotine were assessed by measuring the auditory startle reflex. Startle is a brainstem to spinal cord reflex which has proven useful for studying reflex alterations after pharmacological treatment, as nearly all defined neurotransmitter systems have been shown to interact in modulating the startle responding (Davis, 1987; Davis *et al.*, 1982). CNS hyperexcitability during nicotine withdrawal may be reflected in activation of common neural circuitry involved in the acoustic startle response. For example, cessation of chronic nicotine administration (6 or 10 mg/kg/day for 12 days, respectively) has been shown to produce significant increases in sensorimotor reactivity during the first 4–5 days of withdrawal (Helton *et al.*, 1993). Acute replacement of nicotine attenuates this increase in startle responding seen during cessation following chronic exposure.

LY354740 completely attenuated nicotine (6 mg/kg/day for 12 days, s.c.) withdrawal-enhanced startle responding following both intraperitoneal and oral administration with ED_{50}s of 0.003 and 0.7 mg/kg, respectively (Figs 2 and 4). The activity of LY354740 in this model was stereoselective since the inactive isomer, LY366563 (i.p.), did not attenuate the withdrawal-induced increase in sensorimotor reactivity (Fig. 3). LY354740 blocked potentiated startle responding at doses which produced no changes in acute basal startle (Table 1). In other work, we have found LY354740 to be devoid of benzodiazepine-like side-effects at anxiolytic or drug withdrawal suppressing doses. This includes effects on motor function as measured by spontaneous motor activity, neuromuscular coordination as measured

by rotorod, interaction with sedative hynotics such as hexobarbital, memory impairment as demonstrated in a passive avoidance procedure, or changes in convulsant threshold as demonstrated using electro-convulsive shock (Helton *et al.*, 1997). Other compounds which have been reported to work in this model include serotonin-1A antagonists (Rasmussen *et al.*, 1997) and the CCK-B antagonist LY288513 (Rasmussen *et al.*, 1996). These data indicate that the multiple neurotransmitter systems (i.e. serotonin, glutamate, neuropeptide) play a role in the nicotine-withdrawal phenomenon, and might represent novel approaches to treating smoking cessation in humans. Ultimately, human clinical trials will be needed to determine the relative roles of these different neurotransmitter mechanisms in smoking withdrawal in humans.

Interestingly, nicotine and LY354740, two different classes of pharmacological agents which suppress nicotine withdrawal behavior in rats, protect against conditions of glutamate over-excitation *in vitro* and *in vivo*. Nicotine protects cultured cortical (Akaike *et al.*, 1994) and striatal neurons (Marin *et al.*, 1994) from NMDA receptor-mediated excitotoxic injury *in vitro*. *In vivo*, nicotine also protects against the behavioral and neurotoxic effects of systemic kainic acid (Shytle *et al.*, 1996). Likewise, the group II mGluR agonist (2*S*,1′*R*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV) protects cortical neurons from NMDA- and kainate-mediated neuronal degeneration *in vitro* (Bruno *et al.*, 1995; Buisson *et al.*, 1996), and intraventricular DCG-IV was a reported anticonvulsant and neuroprotectant against *in vivo* kainate in the rat (Miyamoto *et al.*, 1997). Thus, nicotine and group II mGluR agonists have in common the ability to protect against ionotropic glutamate receptor agonist-evoked seizures and neurodegeneration. However, nicotine and group II mGluR agonists have been shown to differentially affect the release of endogenous glutamate. DCG-IV or LY354740 block the enhanced release glutamate *in vitro* (East *et al.*, 1995; Di Iorio *et al.*, 1996) and *in vivo* (Battaglia *et al.*, 1997), whereas nicotine has been reported to enhance glutamate release *in vivo* (Toth, 1996). In the rat hippocampus, nicotine enhances glutamate transmission by a presynaptic action at mossy-fiber terminals (Gray *et al.*, 1996), whereas group II mGluR agonists decrease glutamate release at these same synapses (Kamiya *et al.*, 1996). How these acute effects of nicotine and group II mGluR receptor agonists relate to the ability of these compounds to suppress nicotine withdrawal is not clear.

It is possible that the actions of compounds such as LY354740, which acutely modulate glutamate excitation, may be altered in the nicotine-dependent animals. Glutamatergic neuronal transmission *per se* plays a role in neuronal adaptation to chronic nicotine (Stolerman *et al.*, 1995), since NMDA receptor antagonists have been shown to attenuate the development of tolerance to the locomotor depressant effects of nicotine (Shoaib *et al.*, 1994; Shoaib and Stolerman, 1996). Neuronal adaptive mechanisms which ensue following chronic nicotine have also been shown to alter the cellular actions of nicotine. For example, chronic nicotine has been shown to differentially alter nicotine-evoked release of certain neurotransmitters, increasing the ability of nicotine to induce the release of serotonin and dopamine, but decreasing nicotine-mediated acetylcholine release (Yu and Wecker, 1994). The effects of chronic nicotine on mGluR agonist or nicotine modulation of glutamate excitation (i.e. regulation of glutamate release) are not known.

In conclusion, LY354740 is a novel, systemically active pharmacological agent, representing a new therapeutic class for the potential treatment of human disease states. While LY354740 has been shown to have promising systemic activity in an animal model of nicotine withdrawal, much remains unexplored about this compound including identification of the brain site(s) and cellular mechanism of action responsible for its pharmacological activities. The relevance of the behavioral profile of LY354740 to the nicotine withdrawal state in humans remains to be tested.

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The selective mGlu2/3 receptor agonist LY354740 attenuates morphine-withdrawal-induced activation of locus coeruleus neurons and behavioral signs of morphine withdrawal

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Abstract

Naltrexone-precipitated morphine withdrawal induces hyperactivity of locus coeruleus (LC) neurons, as well as a plethora of behavioral withdrawal signs. Previous research has demonstrated that an increased release of glutamate and activation of AMPA receptors, particularly in the LC, play an important role in opiate withdrawal. LY354740 is a novel Group II metabotropic glutamate mGlu2/3 receptor agonist that decreases the release of glutamate. Therefore, we investigated the effect of LY354740 on naltrexone-precipitated morphine-withdrawal-induced activation of LC neurons and behavioral signs of morphine withdrawal. In in vivo recordings from anesthetized rats, pretreatment with LY354740 (3–30 mg/kg, s.c.) dose-dependently attenuated the morphine-withdrawal-induced activation of LC neurons. In unanesthetized, morphine-dependent animals, pretreatment with LY354740 (3–30 mg/kg, s.c.) dose-dependently suppressed the severity and occurrence of many naltrexone-precipitated morphine-withdrawal signs. These results indicate mGlu2/3 receptor agonists: (1) can attenuate the morphine-withdrawal-induced activation of LC neurons and many behavioral signs of morphine withdrawal; and (2) may have therapeutic effects in man for the treatment of opiate withdrawal.

Keywords: Metabotropic glutamate receptors; Morphine; Withdrawal; Locus coeruleus; Opiates

1. Introduction

The locus coeruleus (LC) is the largest grouping of noradrenergic neurons in the mammalian brain (Dahlstrom and Fuxe, 1965). The cell bodies of the LC are confined to a small area of the pons, but these neurons send extensive projections throughout the neuraxis (Jones and Moore, 1977). These wide ranging projections put the LC in a position to simultaneously influence the activity of a number of brain areas. Hence, the LC has been hypothesized to play a role in a wide variety of behaviors, physiological processes, and brain diseases. In particular, the LC has been shown to play an important role in the expression of opiate withdrawal (Aghajanian, 1978; Rasmussen et al., 1990; Maldonado et al., 1992; Maldonado and Koob, 1993; Rasmussen, 1995). Specifically, the increased release of glutamate and subsequent activation of AMPA receptors is critically involved in opiate withdrawal-induced activation of LC neurons and the behavioral signs of opiate withdrawal (Akaoka and Aston-Jones, 1991; Aghajanian et al., 1994; Rasmussen et al., 1996).

Glutamate receptors have been divided into two broad categories: iontotropic and metabotropic. Iontotropic glutamate receptors contain cation-specific ion channels as a component of their protein complex, while metabotropic glutamate receptors are coupled to G-proteins and modulate intracellular second messenger systems. Thus far, eight different clones for metabotropic glutamate (mGlu) receptors have been isolated (mGlu1-8). Based on agonist interactions, sequence homology, and second messenger coupling, the eight mGlu receptors have been grouped into three large families (Conn and Pin, 1997). Group I mGlu receptors include mGlu1 and mGlu5, Group II mGlu receptors include mGlu2 and mGlu3, and Group III mGlu receptors include mGlu4, 6, 7, and 8. mGlu receptors can differentially modulate synaptic function through both

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pre- and post-synaptic sites (Schoepp and Conn, 1993; O'Leary et al., 1997). Activation of presynaptic Group II and III mGlu receptors decrease the release of glutamate (Pin and Duvoisin, 1994), while activation of presynaptic Group I mGlu receptors can enhance or depress the release of glutamate (Herrero et al., 1992; Gereau and Conn, 1995). Dube and Marshall (1997) demonstrated that mGlu2/3 receptors inhibit excitatory synaptic transmission in LC neurons, possibly by functioning as autoreceptors for excitatory amino acids. In addition to their presynaptic modulation, activation of postsynaptic mGlu1 and 5 receptors stimulate phosphoinositide hydrolysis, while activation of mGlu2, 3, 4, 6, 7 and 8 receptors inhibit cAMP production (Nakanishi, 1992; Schoepp and Conn, 1993). Therefore, mGlu2/3 receptors can influence a variety of glutamatergic dependent processes by either suppressing postsynaptic neuronal activity or inhibiting presynaptic release of glutamate (Nakanishi, 1992; Pin and Duvoisin, 1994).

Recently, it has been shown that i.c.v. administration of the mGlu2/3 receptor agonist DCG-IV and the non-selective mGlu agonist ACPD attenuate some opiate withdrawal signs in rats (Fundytus and Coderre, 1997). However, a limited number of withdrawal symptoms were examined in this study and ACPD and DCG-IV are not systemically active. Further, DCG-IV is not only an mGlu2/3 agonist, but it is also an agonist for mGlu8 and NMDA receptors, as well as an mGlu7 receptor antagonist (Uyama et al., 1997; Breakwell et al., 1997). Thus, the role of mGlu2/3 receptors in morphine withdrawal and the potential therapeutic uses of mGlu receptor ligands in opiate withdrawal has not been fully explored.

LY354740 is a recently discovered selective mGlu2/3 receptor agonist, which shows no significant iontotropic (iGluR4 and iGluR6) nor mGlu1 and 5 or mGlu4, 6, 7 and 8 receptor activities (Monn et al., 1997; Schoepp et al., 1997). LY354740 is systemically active and highly efficacious in animal models of anxiety and nicotine withdrawal (Helton et al., 1997; Helton et al., 1998). In addition, LY354740 has been shown to decrease the evoked release of glutamate in the striatum (Battaglia et al., 1997). Since increased release of glutamate has been shown to play a critical role in the opiate withdrawal, we examined the effect of LY354740, and its inactive enantiomer LY317207, on the opiate withdrawal-induced activation of LC neurons and behavioral signs of opiate withdrawal.

2. Methods

2.1. Opiate dependence and withdrawal

Opiate dependence was induced in male Sprague–Dawley rats (Charles River, 250–350 g) by the subcutaneous pellet implantation method (Way et al., 1969; Blasig et al., 1973). While under halothane anesthesia, animals were implanted with either morphine pellets (NIDA; 75 mg morphine base, 68.5 mg microcrystalline cellulose, 1.5 mg magnesium stearate, 2.5 mg colloidal silicon dioxide) or placebo pellets (NIDA; 150 mg Avicel PH-102, 1.5 mg magnesium stearate, 0.75 mg colloidal silicon dioxide, 1.75 mg purified water). Two pellets were implanted daily for 2 days. Withdrawal was induced 48 h after the last set of pellets was implanted. To insure steady levels of withdrawal throughout the test period, all four pellets were removed one to 2 h before precipitating withdrawal. Withdrawal was induced by administering the opiate antagonist naltrexone HCl (10 mg/kg; Research Biochemical) subcutaneously.

2.2. Behavioral ratings

For the behavioral assessment of opiate withdrawal, animals were studied in clear plexiglass cages (18 × 10 × 8 in) and remained in these cages for the entire study. Animals were adapted to the cage for 15 min, and were then administered a pretreatment of either LY354740, LY317207, or saline (1 ml/kg, s.c.). Naltrexone was administered 15 min after the pretreatment. Twelve behaviors characteristic of the rat opiate abstinence syndrome were assessed (Himmelsbach et al., 1935; Way et al., 1969; Wei, 1973; Blasig et al., 1973; Aceto et al., 1986). The absolute frequency of eight episodic behaviors was recorded and a score was calculated based on multiples of five incidents (0 = no incidents; 1 = 1–5 incidents; 2 = 6–10 incidents; 3 = 11–15 incidents, etc.). Behaviors scored in this manner were teeth chatter (separated by at least 2 s), jumping, wet-dog shakes, writhing, stereotyped head movements, digging, and erections. Chewing (without any matter in the mouth) was similarly scored in multiples of 100 occurrences. Five withdrawal behaviors could not be defined in discrete episodes and the severity of these behaviors was assessed using a four point scale; 0 = absent; 1 = mild; 2 = moderate; 3 = marked. Behaviors rated in this fashion were lacrimation, ptosis, irritability, diarrhea, and salivation. The amount of weight loss was measured at the end of the rating period (i.e. 1 h after the administration of naltrexone) and a score was calculated based on multiples of 5 g (0 = no loss; 1 = 1–5 g; 2 = 6–10 g; 3 = 11–15 g, etc.).

2.3. In vivo electrophysiological recordings

The animals were anesthetized with chloral hydrate (400 mg/kg, i.p.); supplemental doses of anesthetic were administered through a lateral tail vein as needed. Body temperature was maintained at 35–37°C by a heating pad. Rats were mounted in a stereotaxic apparatus and a cisternal drainage was performed to help prevent tissue swelling. A burr hole was made 1.2 mm posterior

to lambda and 1.1 mm lateral to the midline. The recording electrodes were single-barrel glass micropipettes, broken back to a tip diameter of 2–3 μm and filled with a 2 M NaCl solution. Extracellular single-unit recordings were made in the LC. LC cells were encountered 5.5–6.0 mm below the dural surface (at the above coordinates), just ventral to a zone of relative electrical silence (corresponding to the IVth ventricle), and just medial to cells of the mesencephalic nucleus of the Vth nerve (which could be activated by depression of the mandible). LC cells were identified by their long duration action potential (> 2 ms), characteristic positive-negative waveform, slow and somewhat regular firing pattern, slow firing rate (0.5–5 Hz), and short-latency burst of excitation followed by an extended (1–2 s) quiescent period following compression of the contralateral hindpaw. Recordings of stable, spontaneous firing rates were obtained from each neuron for at least 3 min. The spontaneous rate of different cells was sampled throughout the LC for 15 min before and after the administration of LY354740 or LY317207 and for 60 min after the administration of naltrexone.

2.4. Statistical analysis

Electrophysiological results were analyzed with a two-way ANOVA coupled with Student's post-hoc *t*-test. Composite withdrawal score results were analyzed with a repeated measures MANOVA coupled with Student's post-hoc *t*-test. Individual behavior scores were analyzed with one-way ANOVAs.

3. Results

3.1. Electrophysiological recordings

Pretreatment with LY354740, but not LY317207, significantly attenuated the morphine-withdrawal-induced activation of LC neurons (ANOVA; $F = 7.45$, $P < 0.0001$; Fig. 1). The basal firing rates of LC cells in animals implanted with morphine pellets were significantly lower than in animals receiving placebo pellets ($P < 0.05$), indicating an incomplete development of tolerance to morphine at the time of testing (Fig. 1; baseline). Pretreatment with LY354740 did not alter the firing rates of LC neurons in either morphine or placebo implanted animals (Fig. 1; 0 min post naltrexone). After naltrexone administration, the firing rates of LC cells were significantly elevated for both saline ($P < 0.05$) and LY317207 ($P < 0.05$) pretreated, morphine-dependent animals at 15, 30, 45, and 60 min post naltrexone, but not in placebo-implanted animals (Fig. 1). LY317207 and saline pretreated morphine-dependent animals showed no significant differences in LC firing rates at all time points. Following naltrexone administration, pretreatment with 3 mg/kg LY354740 significantly ($P < 0.05$) attenuated the morphine-withdrawal induced activation of LC firing rates at 15, 30, and 60 min. Pretreatment with 10 mg/kg and 30 mg/kg LY354740 significantly ($P < 0.05$) attenuated the morphine-withdrawal induced activation of LC firing rates at 15, 30, 45 and 60 min time intervals. After naltrexone, the LC firing rates of morphine-dependent rats pretreated with 10 and 30 mg/kg LY354740 were not significantly elevated from placebo implanted animals.

3.2. Behavioral ratings

Pretreatment with LY354740, but not LY317207, significantly attenuated morphine withdrawal signs induced by the administration of naltrexone in morphine dependent animals (MANOVA; $F = 6.11$, $P < 0.0001$; Fig. 2). Many individual withdrawal scores which contributed to the total withdrawal scores were significantly reduced in a dose-dependent manner. Digging was significantly ($P < 0.05$) decreased by all doses of LY354740. Pretreatment with 10 and 30 mg/kg LY354740 significantly ($P < 0.05$) suppressed writhes, salivation, diarrhea, and chews. The occurrence of wet-dog shakes and ptsosis were significantly ($P < 0.05$)

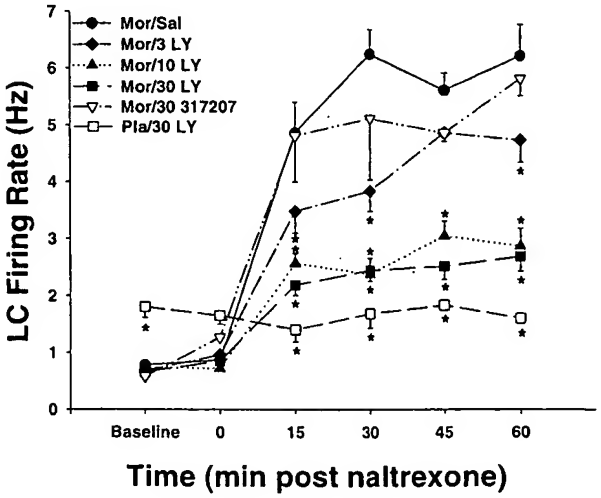

Fig. 1. The activity of locus coeruleus (LC) neurons during naltrexone-precipitated withdrawal in morphine (Mor)-dependent, anesthetized rats pretreated with saline (Sal) (Mor/Sal; ●), 3 mg/kg LY354740 (LY) (Mor/3 LY; ◆), 10 mg/kg LY (Mor/10 LY; ▲), 30 mg/kg LY (Mor/30 LY; ■), or 30 mg/kg 317207 mg/kg (Mor/30 317207; △). The activity of LC neurons in animals receiving placebo (Pla) pellets and treatment with 30 mg/kg LY followed by naltrexone is also shown (Pla/30 LY; □). Values are expressed as mean + SE (some error bars omitted for clarity; $n = 4$ to 36 neurons at each time point). Significant differences ($P < 0.05$) are denoted by an asterisk (*).

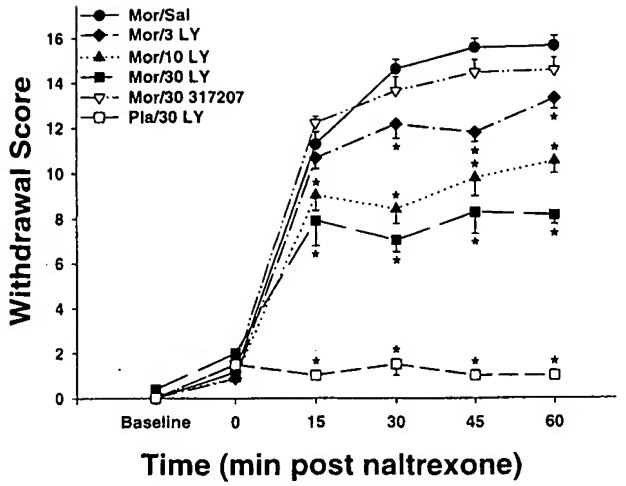

Fig. 2. Effects of pretreatment with saline (Mor/Sal; ●; $n = 8$), 3 mg/kg LY (Mor/3 LY; ◆; $n = 8$), 10 mg/kg LY (Mor/10 LY; ▲; $n = 8$), 30 mg/kg LY (Mor/30 LY; ■; $n = 8$), or 30 mg/kg 317207 mg/kg (Mor/30 317207; △; $n = 8$) on naltrexone-precipitated withdrawal signs in morphine-dependent rats. Values are expressed as mean ± SE (some error bars omitted for clarity). Significant differences ($P < 0.05$) are denoted by an asterisk (*).

suppressed by pretreatment with 30 mg/kg LY354740. Chatters, jumps, lacrimination, irritability, erections, and weight loss were not significantly reduced by any dose of LY354740 (Table 1). No individual withdrawal behaviors were significantly reduced by pretreatment with LY317207. Doses of LY354740 up to 100 mg/kg had no significant effect on locomotor activity or the righting reflex and produced no signs of sedation (data not shown).

4. Discussion

The results of this study demonstrate that activation of mGlu2/3 receptors can have a strong influence on opiate withdrawal. In morphine dependent rats, LY354740, but not its inactive isomer LY317207, significantly reduced naltrexone-precipitated activation of LC neurons. The typical 5 to 6-fold increase in LC neuronal firing rates seen during morphine withdrawal was dose-dependently suppressed by acute pretreatment with LY354740. This finding is consistent with previous research demonstrating that presynaptic mGlu receptors can act as glutamate autoreceptors to inhibit activation of LC neurons (Dube and Marshall, 1997). Since there is an increase release of glutamate in the LC during morphine withdrawal (Aghajanian et al., 1994), and LY354740 can reduce the veratridine-stimulated release of glutamate in vivo (Battaglia et al., 1997), it seems likely that LY354740 attenuates the morphine withdrawal induced activation of LC neurons, at least in part, by decreasing the release of glutamate. However, presynaptic Group II mGlu receptors have also been shown to reduce transmission at inhibitory GABA synapses (Salt and Eaton, 1995). Since the LC receives GABA containing afferents (Ennis and Aston-Jones, 1989), LY354740 may also affect the release of GABA in the LC. In addition, indirect effects of presynaptic mGlu receptors on the release of other neurotransmitters in the LC may also play a role in the effects of LY354740. Further studies are needed to determine if LY354740 affects the release of glutamate, GABA, and/or other neurotransmitters in the LC during opiate withdrawal.

LY354740, but not LY317207, dose-dependently inhibited the overall withdrawal score, as well as several individual withdrawal behaviors seen during naltrexone-precipitated morphine withdrawal. The maximal decrease in the overall withdrawal score produced by LY354740 is comparable to the maximal suppression of opiate withdrawal achieved with NMDA and AMPA antagonists (Rasmussen et al., 1996). However, unlike NMDA and AMPA antagonists, even very high doses of LY354740 did not produce sedation or ataxia. In addition to reducing total withdrawal scores, LY354740 significantly suppressed many of the individual withdrawal symptoms. Pretreatment with 10 mg/kg, i.p., decreased writhes, digging, salivation, diarrhea and chews. At the highest dose tested (30 mg/kg, i.p.), wet dog shakes and ptsosis were also significantly reduced. Thus, LY354740 suppressed many of the same withdrawal behaviors as NMDA and AMPA antagonists (Rasmussen et al., 1991; Rasmussen et al., 1996).

While the presynaptic actions of LY354740 in the LC are likely to play a major role in its suppression of morphine withdrawal symptoms, the activation of postsynaptic mGlu2/3 receptors in the LC may also be involved. Recent evidence has shown that mGlu2/3 agonists, including LY354740, inhibit cAMP formation and adenylate cyclase (AC) activity via postsynaptic receptors (Schaffhauser et al., 1997). Upregulation of cAMP and AC pathways in the LC play an important role in the development and expression of morphine dependence (Nestler, 1996; Nestler and Aghajanian, 1997). For example, chronic morphine administration increases levels of AC and cAMP-dependent protein kinases activity in the LC and intra-LC administration of cAMP-dependent protein kinase inhibitors attenuates opiate withdrawal (Lane-Ladd et al., 1997; Punch et al., 1997). Therefore, LY354740 may attenuate the morphine withdrawal-induced activation of LC neurons by not only inhibiting the release of glutamate, but also by reducing the production of cAMP in LC neurons.

In addition to effects in the LC, actions of LY354740 in other brain areas may play a role in its suppression of morphine withdrawal behaviors. Other potential sites of action of LY354740 include those with the highest densities of mGlu2/3 receptors, including the cerebral cortex, olfactory areas, hippocampus, substantia nigra,

Table 1
Effect of pretreatment with saline, LY354740, or LY317207 on individual withdrawal signs[a]

Behaviors	Saline		3 mg/kg		10 mg/kg		30 mg/kg		317207	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Chatter	4.42	1.39	5.86	1.60	5.88	1.37	8.75	3.21	5.70	1.07
Jump	1.00	0.44	1.50	0.53	0.00	0.00	0.13	0.13	1.60	0.52
Writh	6.25	1.30	4.50	1.65	0.88*	0.52	0.25*	0.16	10.50	2.85
Dog shake	11.25	2.36	7.25	1.78	11.38	3.47	4.50*	1.43	9.40	2.36
Ptosis	9.75	0.22	8.75	0.53	8.50	0.53	7.50*	1.00	9.20	0.57
Digging	46.17	10.42	21.63*	8.20	19.00*	7.40	17.50*	4.51	27.20	7.33
Lacrimination	0.83	0.34	1.00	0.38	0.38	0.18	0.50	0.19	0.70	0.52
Salivation	7.42	0.42	7.00	0.65	4.50*	0.71	4.13*	0.30	7.10	0.59
Irritability	5.25	0.60	4.75	0.86	5.25	0.60	3.38	0.65	5.70	0.76
Erection	1.67	0.57	2.00	0.65	1.87	0.44	2.87	1.91	2.30	0.60
Diarrhea	7.25	0.52	6.00	0.57	2.75*	0.25	2.88*	0.61	7.80	0.33
Chews	528.17	80.11	431.50	124.63	260.50*	36.93	247.63*	56.36	540.50	47.57
Weight (g)	21.67	1.83	20.50	1.41	18.75	1.47	18.75	1.62	24.60	1.83

[a] Values represent total expression (mean.± SE) of each behavior for the 60-min period following naltrexone administration.
* Significant differences ($P<0.05$).

habenula, fimbria, interpeduncular nucleus, inferior olive, cerebellum, and spinal cord (Petralia et al., 1995). Importantly, mGlu2/3 receptors are located in some areas thought to be involved in opiate withdrawal behaviors such as the hypothalamus, amygdala, periaquaductal grey area, and spinal cord (Maldonado et al., 1992; Petralia et al., 1995). One potentially important site for the actions of LY354740 during morphine withdrawal is the nucleus paragigantocellularis (PGi). The PGi contains mGlu2/3 receptors, sends a major glutamatergic afferent to the LC, and lesions of the PGi reduce morphine withdrawal symptoms (Ennis and Aston-Jones, 1988; Rasmussen and Aghajanian, 1989; Petralia et al., 1995). Thus, activation of mGlu2/3 receptors in the PGi may reduce subsequent release of glutamate in the LC and attenuate activation of LC neurons. Additional experiments will be needed to address this hypothesis.

The relative contribution of mGlu2 versus mGlu3 receptors to the action of LY354740 can not be determined from this study. However, given that LY354740 has a 6-fold higher affinity for mGlu2 than mGlu3 receptors (Schoepp et al., 1997) and that mGlu2 and 3 receptors have a differential distribution in the brain (Ohishi et al., 1993a,b), activation of mGlu2 and mGlu3 receptors may have different effects on morphine withdrawal. Additional studies with compounds that have greater selectively for mGlu2 or mGlu3 receptors will be needed to elucidate the role of each receptor subtype in opiate withdrawal.

In conclusion, the selective mGlu2/3 agonist, LY354740, but not its inactive isomer LY317207, suppressed the morphine-withdrawal-induced activation of LC neurons and many morphine withdrawal symptoms. These results indicate that mGlu2/3 receptors can have a strong influence on opiate withdrawal and that LY354740, and other mGlu2/3 agonists, may have therapeutic potential for the treatment of opiate withdrawal.

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Effect of activity at metabotropic, as well as ionotropic (NMDA), glutamate receptors on morphine dependence

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1 The contribution of various excitatory amino acid (EAA) receptors (NMDA, AMPA/kainate and metabotropic) in the brain to the development of morphine dependence was examined. This was performed by measuring the severity of the precipitated withdrawal syndrome following chronic subcutaneous (s.c.) morphine and intracerebroventricular (i.c.v.) EAA antagonist treatment.

2 Continuous subcutaneous (s.c.) treatment with morphine sulphate (36.65 μmol day^{-1}) produced an intense and reliable naloxone-precipitated withdrawal syndrome.

3 Chronic i.c.v. treatment with antagonists selective for metabotropic and NMDA receptors, but not AMPA/kainate receptors, significantly attenuated abstinence symptoms. Conversely, EAA antagonists had very little effect on non-withdrawal behaviours.

4 These results suggest that, as well as changes elicited by activation of NMDA receptors, metabotropic receptors and intracellular changes in the phosphatidylinositol (PI) second-messenger system or the cyclic adenosine 3′,5′-monophosphate (cAMP) second messenger system, to which EAA metabotropic receptors are linked, may be involved in the development of opioid dependence with chronic morphine treatment.

Keywords: Opioid; morphine; dependence; glutamate; metabotropic glutamate receptor; AMPA; kainate; NMDA; abstinence syndrome; excitatory amino acids

Introduction

Although opioid drugs such as morphine are widely used for the management of pain, their clinical usefulness is limited by the development of tolerance and dependence that occurs with their chronic use. Tolerance is indicated by a decreased efficacy of the drug with repeated administration, and results in a need to increase the morphine dose in order to achieve the desired analgesic effect. Dependence is a continued need for the drug to maintain a state of physiological equilibrium, and leads to an aversive withdrawal or abstinence syndrome when morphine administration is terminated. Recently, it has been demonstrated that co-administration of *N*-methyl-D-aspartate (NMDA) receptor antagonists attenuates the development of morphine tolerance and dependence (Marek *et al.*, 1991a,b; Trujillo & Akil, 1991). Since the endogenous excitatory amino acid (EAA) glutamate activates NMDA receptors, it is likely that glutamate contributes to the development of these phenomena. In addition to NMDA receptors, glutamate acts at at least two other types of ionotropic receptors: receptors at which α-2-amino-3-(hydroxy-5-methylisoxazol-4yl)propanoic acid (AMPA) is a selective agonist and receptors at which kainate is a selective agonist; and metabotropic receptors (Mayer & Westbrook, 1987a; Monaghan *et al.*, 1989). While a role for the NMDA receptor has already been suggested, the specific contribution of these other receptors to behavioural indices of opioid tolerance and dependence has not been investigated.

The NMDA receptor gates a cation channel that is permeable to Ca^{2+} and Na^{+} (MacDermott *et al.*, 1986; Mayer *et al.*, 1987) and is gated in a voltage-dependent fashion by Mg^{2+}(Mayer *et al.*, 1984; Nowak *et al.*, 1984). It is the voltage-dependent Ca^{2+} permeability of the NMDA receptor that is thought to be necessary for use-dependent synaptic plasticity (Cotman *et al.*, 1988) and may be critical for the development of neuronal changes that mediate opioid tolerance and dependence (Marek *et al.*, 1991a,b; Trujillo & Akil, 1991). AMPA and kainate receptors gate cation channels that are permeable to Na^{+}, but for the most part have negligible permeability to Ca^{2+} (Mayer & Westbrook, 1987b; Murphy & Miller, 1989). However, AMPA/kainate receptors that exhibit Ca^{2+} permeability have recently been cloned (Miller, 1991; Sommer & Seeburg, 1992). Traditionally, AMPA/kainate receptors are thought to be involved in the mediation of rapid excitatory responses to EAA transmitters (Kiskin *et al.*, 1986; Jonas & Sakmann, 1992) and may contribute to neuronal plasticity by relieving the NMDA receptor of its voltage-dependent block by Mg^{2+}. Unlike ionotropic receptors, metabotropic glutamate receptors are not linked to cation channels. Instead they are coupled directly to the cell membrane by a G protein (Sladeczek *et al.*, 1985; Sugiyama *et al.*, 1987). Several subtypes of metabotropic glutamate receptors have recently been cloned. Some subtypes affect phosphatidylinositol (PI) hydrolysis (mGluR1α, mGluR1β and mGluR5), while others affect the production of adenosine 3′,5′-cyclic monophosphate (cAMP) (mGluR2, mGluR3, mGluR4) (Schoepp & Conn, 1993). Activity at metabotropic receptors coupled to the PI system activates phospholipase C, which catalyses phosphatidylinositol hydrolysis, leading to the production of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Ambrosini & Meldolesi, 1989; Manzoni *et al.*, 1990; Schoepp & Conn, 1993). Activity at metabotropic receptors coupled to the cAMP second messenger system generally leads to decreased production of cAMP, although activation of mGluR1α stimulates an increase in cAMP (Schoepp & Conn, 1993). Through the increased production of intracellular messengers associated with PI hydrolysis, or decreased production of cAMP, metabotropic receptor activation may play an important role in the long-term effects mediated by glutamate (Nicoletti *et al.*, 1991), and like NMDA receptors may be critical to the development of neuronal changes mediating opioid tolerance and dependence.

In the present study we have investigated the contribution of various EAA receptor subtypes in the brain to the

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development of opioid dependence. This purpose was achieved by examining the effects of the intracerebroventricular (i.c.v.) administration of selective EAA receptor antagonists concurrently with the chronic subcutaneous (s.c.) administration of morphine. NMDA receptors were antagonized with the non-competitive antagonist 5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine hydrogen maleate (MK-801) (Wong *et al.*, 1986); AMPA/kainate receptors were antagonized with 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine-hydrochloride (GYKI 52466) (Donevan & Rogawski, 1993). Although distinct families of high-affinity AMPA and kainate receptors have been isolated, the functional distinction between these receptors is not entirely clear (Barnard & Henley, 1990). Consequently, pharmacological investigations with receptor antagonists are limited to investigations of non-selective AMPA/kainate receptor effects. Metabotropic receptors were antagonized with the highly selective antagonist (*S*)-4-carboxyphenylglycine [(*S*)-4C-PG] (Birse *et al.*, 1993; Eaton *et al.*, 1993) and a more commonly used, yet less selective antagonist, L-2-amino-3-phosphonopropanoic acid (L-AP3) (Schoepp *et al.*, 1990; Birse *et al.*, 1993).

Methods

Subjects and surgery

Subjects were male Long Evans rats (280–350 g). The rats were housed 2–3 per cage, on a 12:12 h light–dark cycle (lights on at 06:00 h), with food and water available *ad libitum*.

On day 0 rats were anaesthetized with sodium pentobarbital (Somnotol, MTC Pharmaceuticals, 60 mg kg^{-1}), and 23 gauge stainless steel cannulae, attached to model 2001 Alzet osmotic mini-pumps filled with one of the EAA antagonist solutions or saline, were implanted stereotaxically in the lateral ventrical (AP = −1.3 mm and L = −1.8 mm from bregma and V = −3.8 mm from the top of the skull; Paxinos & Watson, 1986). While the rats were still under pentobarbital anaesthesia, one unprimed (i.e. not yet pumping) model 2ML1 Alzet pump containing 60 mg ml^{-1} morphine sulphate solution was implanted s.c. on the back. These morphine-containing pumps started pumping the morphine solution approximately 2–4 h following implantation. On the following day, day 1, rats were briefly anaesthetized with halothane and a second unprimed model 2ML1 Alzet pump containing 60 mg ml^{-1} morphine sulphate solution was implanted s.c. on the back. This two day pump implantation procedure was used to reduce the risk of mortality resulting from the accumulation of lethal systemic morphine concentrations prior to any tolerance development. To assess the effects of chronic i.c.v. EAA antagonist treatment on behaviour in rats not dependent on morphine, some rats were given vehicle or 40 nmol day^{-1} L-AP3, (*S*)-4C-PG, MK-801 or GYKI 52466 without concurrent morphine treatment.

Drugs

MK-801, L-AP3 and GYKI 52466 were obtained from Research Biochemicals, (Natick, MA, U.S.A.), while (*S*)-4C-PG was purchased from Tocris Neuramin (Bristol, U.K.). EAA antagonists were continuously infused at a rate of 1 µl h^{-1} in the following concentrations: 1.6 nmol day^{-1}, 8 nmol day^{-1} and 40 nmol day^{-1}. Morphine sulphate (Sabex, Montreal, Canada) was continuously delivered at a rate of 10 µl h^{-1} for a total dose of 36.65 µmol day^{-1} morphine sulphate.

Withdrawal measurement

Precipitated abstinence symptoms were assessed on the seventh day of treatment (while all pumps were still delivering antagonists and morphine) after injection of naloxone (1 mg kg^{-1} s.c.). For 10 min before and 40 min after naloxone injection, the withdrawal symptoms were assessed by measuring the amount of time spent teeth chattering and writhing, as well as by counting jumps and wet dog shakes (Bläsig *et al.*, 1973). The time spent in non-withdrawal behaviours (ambulating, rearing, grooming and resting) was also measured for comparison, for 10 min before and after the injection of naloxone, in rats treated with i.c.v. EAA antagonists either alone or with s.c. morphine.

Statistical analysis

Timed withdrawal behaviours (teeth chattering, writhing) were analysed using ANOVA, followed by *post hoc* tests on significant main effects. Counted withdrawal behaviours (number of jumps and wet dog shakes) were analysed using a Kruskal–Wallis ANOVA for non-parametric data, followed by Mann–Whitney *U*-tests on significant main effects.

The effect of EAA antagonist treatment on non-withdrawal behaviours (ambulating, rearing, grooming and resting) was assessed by comparing the first two time blocks (i.e. 10 min prior to naloxone injection and 10 min after naloxone injection) for rats in each treatment group. Planned comparisons were used to analyse differences in the proportion of time spent in each timed non-withdrawal and the two timed withdrawal behaviours during these two time blocks across the different treatment conditions.

Results

Administration of 36.65 µmol day^{-1} s.c. morphine sulphate by Alzet pump produced an intense and reliable naloxone-precipitated abstinence syndrome which was evidenced by the occurrence of teeth chattering, writhing, jumping and wet dog shaking. As indicated in Figure 1a, the metabotropic receptor antagonists (*S*)-4C-PG and L-AP3 significantly decreased the occurrence of timed abstinence symptoms (teeth chattering and writhing). The effect for (*S*)-4C-PG appeared to be dose dependent, with the highest dose, 40 nmol day^{-1}, producing the greatest reduction in teeth chattering and writhing. L-AP3 was most effective at 8 nmol day^{-1}. Figure 1b shows the amount of time spent teeth chattering and writhing for rats treated with MK-801 and GYKI 52466. The NMDA receptor antagonist MK-801 significantly decreased the time spent in withdrawal at all doses used. The AMPA/kainate receptor antagonist GYKI 52466 did not affect the amount of time spent in withdrawal at any of the doses used.

Figure 2 illustrates the frequency of the counted abstinence symptoms, jumps and wet dog shakes. Although MK-801 tended to *increase* the number of jumps and wet dog shakes at the high dose (40 nmol day^{-1}), none of the i.c.v. EAA antagonist treatments *significantly* affected the number of jumps and wet dog shakes.

Figure 3 shows the percentage of time spent in each of the timed behaviours during the 10 min prior to naloxone administration and during the 10 min following naloxone for rats in each i.c.v. treatment group either with or without concurrent morphine treatment. As can be seen in Figure 3a and b, prior to the injection of naloxone, rats in all i.c.v. treatment groups, with or without morphine, behaved very similarly, with the only differences being more grooming in L-AP3 treated rats than in saline treated rats. In addition, saline-treated rats that were also given morphine reared more that rats given i.c.v. saline alone. Although activity levels (ambulating and rearing) were lower and resting was generally higher after the injection of naloxone, rats given i.c.v. EAA antagonists without morphine still behaved very similarly to rats given i.c.v. saline without morphine. The only differences observed were an increase in grooming in L-AP3-treated rats and increased activity in GYKI 52466-

treated rats, as evidenced by increased ambulating, rearing and grooming and decreased resting. As expected, rats dependent on morphine showed significantly more naloxone-precipitated withdrawal, with a resultant decrease in non-withdrawal behaviours, than rats given i.c.v. treatments alone. In morphine-dependent rats, time in withdrawal was significantly less in L-AP3-, (*S*)-4C-PG- and MK-801-treated rats, which coincided with an increase in ambulation in (*S*)-4C-PG- and MK-801-treated rats.

Discussion

The present results demonstrate that concurrent treatment of rats with various EAA antagonists and chronic morphine leads to a decrease in various symptoms of morphine withdrawal. The NMDA receptor antagonist MK-801 and the metabotropic receptor antagonists (*S*)-4C-PG and L-AP3 were all effective at decreasing the amount of time spent exhibiting the withdrawal symptoms of teeth chattering and writhing, while the AMPA/kainate receptor antagonist GYKI 52466 had no effect. Although all but the AMPA/kainate receptor antagonist significantly reduced the timed withdrawal symptoms, none of the EAA antagonists significantly affected the counted withdrawal symptoms (i.e. jumping and wet dog shakes). It has been suggested that withdrawal symptoms such as jumping are mediated primarily by structures around the fourth ventricle, as evidenced by focal brain micro-injections of naltrexone and levallorphan in morphine-dependent rats (Laschka *et al.*, 1976; Koob *et al.*, 1992). In the present study, EAA antagonists were infused in very small volumes into the lateral ventricle, thus it is possible that the counted symptoms were not affected because the drugs were unable to diffuse to the appropriate brain structures around the fourth ventricle.

There was very little effect of i.c.v. EAA antagonist treatment on non-withdrawal behaviours, except for a general increase in grooming in rats given L-AP3 and increased activity in GYKI 52466-treated rats given naloxone. In general, rats given i.c.v. treatment alone rested more during the second 10 min (i.e. after naloxone), with concurrent decreases in ambulation and rearing. This is probably *not* an effect of naloxone, but rather because they had adequately explored the test box and were comfortable in the environment (a phenomenon common in untreated rats). Rats treated chronically with morphine exhibited withdrawal following the injection of naloxone, which caused a subsequent decrease in other timed behaviours. L-AP3, (*S*)-4C-PG and MK-801 all decreased withdrawal compared with saline in morphine-treated rats, with a resultant increase in ambulation in rats given MK-801 + morphine and (*S*)-4C-PG + morphine. The decrease in withdrawal allows for more time to be spent in other behaviours.

Although we have no explanation for why L-AP3 would increase grooming, it appears to be a robust effect since it occurred in every condition except in rats going through withdrawal (i.e. morphine + naloxone). The increase in activity produced by GYKI 52466 appears to be less robust since it occurred only in the group also treated with naloxone. Nonetheless, it is not expected that these behavioural effects of L-AP3 or GYKI 52466 alter our conclusions about the effects of EAA antagonists on withdrawal behaviour. GYKI 52466 did not significantly affect withdrawal, and the effects of the metabotropic receptor antagonist L-AP3 were confirmed by another metabotropic antagonist (*S*)-4C-PG, which did not significantly influence non-withdrawal behaviours. Therefore, we propose that EAA antagonist treatment affects the development of dependence directly, and not indirectly by interfering with the measurement of the withdrawal behaviours.

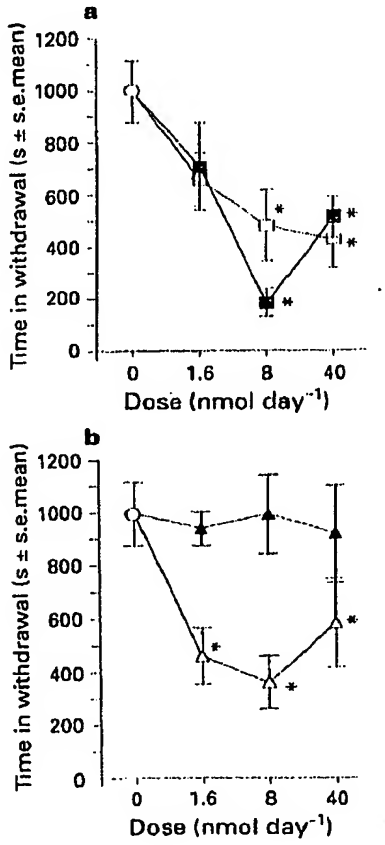

Figure 1 Mean time spent exhibiting withdrawal (teeth chattering and writhing) during the 40 min withdrawal period in rats given chronic s.c. morphine and i.c.v. treatment with the metabotropic receptor antagonists (*S*)-4C-PG (□) ($n = 5-10$ per dose) and L-AP3 (■) ($n = 4-10$ per dose) (a), the NMDA receptor antagonist MK-801 (△) ($n = 4-10$ per dose) (b), and the AMPA/kainate receptor antagonist GYKI 52466 (▲) ($n = 4-10$ per dose) (b). ANOVA indicated significant effects of (*S*)-4C-PG [$F_{(3,22)} = 5.124$, $P < 0.001$], L-AP3 [$F_{(3,23)} = 12.107$, $P < 0.001$] and MK-801 [$F_{(3,21)} = 6.222$, $P < 6.222$, $P < 0.01$], but not GYKI 52466 [$F_{(3,20)} = 0.071$, $P > 0.05$]. Significant differences from the control group are indicated by asterisks (*$P < 0.05$, LSD *t*-test).

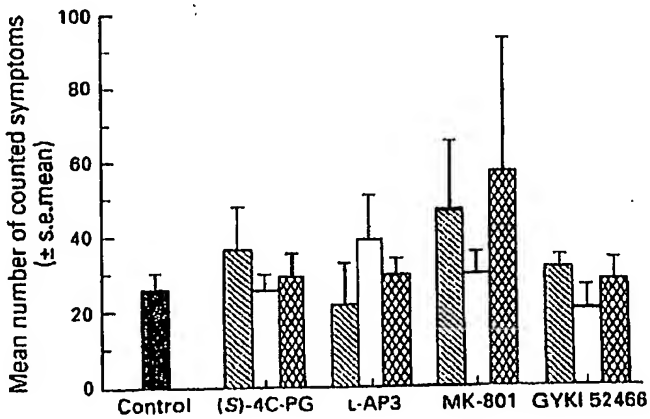

Figure 2 Frequency of counted symptoms (jumps and wet dog shakes) during the 40 min withdrawal period for rats given chronic s.c. morphine and i.c.v. treatment with the metabotropic receptor antagonists (*S*)-4C-PG ($n = 5-10$ per dose) or L-AP3 ($n = 4-10$ per dose), the NMDA receptor antagonist MK-801 ($n = 4-10$ per dose) or the AMPA/kainate receptor antagonist GYKI 52466 ($n = 4-10$ per dose). Kruskal–Wallis ANOVA for non-parametric data revealed no significant effects of any of the EAA antagonists. (*S*)-4C-PG, $H_{(3,22)} = 0.803$, $P > 0.05$; MK-801, $H_{(3,21)} = 0.747$, $P > 0.05$; GYKI 52466, $H_{(3,20)} = 2.580$, $P > 0.05$; L-AP3, $H_{(3,23)} = 2.142$, $P > 0.05$. Solid columns, 0 nmol day^{-1}; diagonal hatched columns, 1.6 nmol day^{-1}; open columns, 8 nmol day^{-1}; cross-hatched columns, 40 nmol day^{-1}.

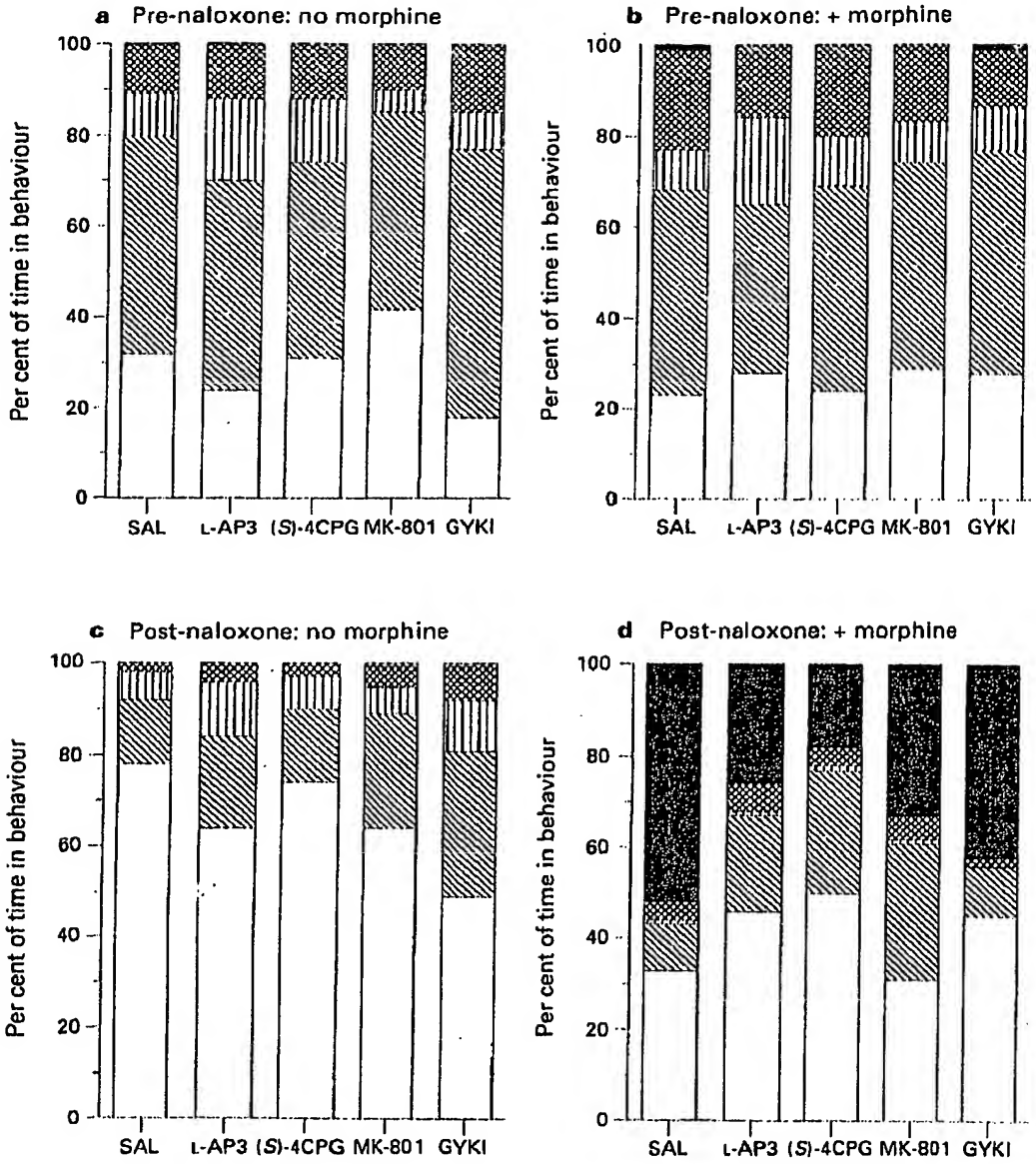

Figure 3 Percentage of time in non-withdrawal and withdrawal behaviours in rats treated chronically with either saline or 40 nmol day^{-1} L-AP3, (*S*)-4C-PG, MK-801 or GYKI 52466 (EAA antagonists) i.c.v. alone (i.e. no s.c. morphine; $n = 6-8$ per i.c.v. treatment) (a) or with s.c. morphine ($n = 4-10$ per i.c.v. treatment) (b) during the 10 min *prior* to the injection of naloxone; and rats given chronic i.c.v. saline or EAA antagonists alone (c) or with s.c. morphine (d) during the 10 min *after* the injection of naloxone. Prior to the injection of naloxone, rats in all i.c.v. treatment groups, with or without morphine, behaved very similarly, with the only differences being increased grooming in L-AP3-treated rats (planned comparison$_{(1,52)}$, $P<0.05$) (a and b). With the addition of morphine, saline-treated rats exhibited more rearing than rats not treated with morphine (planned comparison$_{(1,52)}$, $P<0.05$) (b). After the injection of naloxone, rats not dependent on morphine generally rested more than in the first 10 min. Non-dependent rats in all i.c.v. treatment groups behaved similarly, with the only differences being more grooming in L-AP3-treated rats (planned comparison$_{(1,52)}$, $P<0.05$) (c) and more activity in GYKI 52466-treated rats as evidenced by significantly more ambulating (diagonal hatched columns), rearing (cross-hatched columns) and grooming (vertically hatched columns) and significantly less resting (open columns) (planned comparison$_{(1,52)}$, $P<0.05$) (c). Morphine-dependent rats showed a significant increase in withdrawal behaviours (solid columns) regardless of i.c.v. treatment after the injection of naloxone (planned comparison$_{(1,52)}$, $P<0.05$) (d). However, L-AP3, (*S*)-4C-PG and MK-801 all significantly decreased the percentage of time spent in withdrawal behviours, with a concurrent increase in ambulation in (*S*)-4C-PG- and MK-801-treated rats (planned comparison$_{(1,52)}$, $P<0.05$) (d).

Previously, it has been observed that concurrent treatment of rats with the non-selective EAA antagonist kynurenic acid (Marek *et al.*, 1991a) or the non-competitive NMDA antagonist MK-801 (Marek *et al.*, 1991b; Trujillo & Akil, 1991) with daily injections of morphine attenuated the development of tolerance to morphine's analgesic effects. MK-801 also alleviated the severity of some symptoms of the precipitated withdrawal syndrome (Trujillo & Akil, 1991). Furthermore, some investigators have found that acute treatment with kynurenic acid and MK-801 only on the day of testing (i.e. not concurrently with morphine) is effective in decreasing the severity of some withdrawal symptoms (Rassmussen *et al.*, 1991a,b; Tanganelli *et al.*, 1991), while other did not find this acute administration effective (Trujillo & Akil, 1991). While each of these above studies assessed the effects of *systemically* administered EAA antagonists, in additional experiments (unpublished data), we have found that acute *i.c.v.* injections of EAA antagonists on day 7 prior to precipitation of withdrawal failed to attenuate severity of abstinence symptoms, lending support to the latter group.

Hyperactivity in the locus coeruleus (LC) has been shown to be correlated with the morphine withdrawal syndrome (Aghajanian, 1978; Valentino & Wehby, 1989). Central administration of non-specific, NMDA-selective and AMPA

kainate-selective EAA antagonists into either the lateral ventricle or the locus coeruleus has been found to decrease the hyperactivity of locus coeruleus neurones during precipitated morphine withdrawal (Akaoka & Aston-Jones, 1991), with the best effects produced by the non-specific EAA antagonist. Conversely, systemic administration of selective NMDA receptor antagonists was unable to affect the hyperactivity of locus coeruleus neurones during precipitated morphine withdrawal (Rasmussen *et al.*, 1991a).

The present data indicate that chronic i.c.v. administration of the selective metabotropic EAA receptor antagonists (*S*)-4C-PG and L-AP3 was at least as effective in attenuating the severity of the morphine withdrawal syndrome as antagonists selective for the NMDA receptors. It is noteworthy that the most effective treatment was 8 nmol day^{-1} L-AP3. This strong effect of L-AP3 may have resulted from a possible additive effect of L-AP3 at both metabotropic and NMDA receptors. There is evidence that L-AP3 may have non-selective effects at the NMDA receptor as well as its proposed major action at the metabotropic receptor (Birse *et al.*, 1993). The significant dose-dependent effects of the highly selective metabotropic receptor antagonist (*S*)-4C-PG, however, do suggest an important role of metabotropic glutamate receptors in the development of morphine dependence. The failure of GYKI 52466 to influence precipitated withdrawal suggests that AMPA/kainate receptors do not play an important role in morphine dependence. Although it is possible that GYKI 52466's ineffectiveness could be explained by rapid metabolism *in vivo*, this is unlikely because the drug was chronically infused. Furthermore, it has been demonstrated, using an osmotic pump infusion method similar to that used in the present study, that GYKI 52466 is as effective at attenuating excitatory amino acid induced seizures on the 14th day of infusion as it is on the third day of infusion (Steppuhn & Turski, 1993).

Activity at specific metabotropic receptor subtypes stimulates phosphatidylinositol (PI) hydrolysis and leads to the production of the intracellular messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Ambrosini & Meldolesi, 1989; Manzoni *et al.*, 1990; Schoepp & Conn, 1993). Chronic opioid use may alter production of these intracellular messengers and thus elicit long-term changes which contribute to opioid tolerance and dependence. There is evidence that acute morphine treatment stimulates PI hydrolysis (Raffa & Martinez, 1992), while chronic morphine treatment inhibits PI hydrolysis (Dixon *et al.*, 1990). (*S*)-4C-PG and L-AP3 antagonism of the metabotropic glutamate receptor during morphine treatment may prevent cellular changes associated with persistent phosphatidylinositol hydrolysis, and consequently reduce withdrawal symptoms that are associated with these cellular changes. Other metabotropic receptor subtypes inhibit production of cAMP (Schoepp & Conn, 1993). It is well established that both acute and chronic opioid use also affect the production of cAMP (Collier, 1980, 1983; Sharma *et al.*, 1975). Thus it is possible that (*S*)-4C-PG and L-AP3 antagonism of metabotropic receptors prevented changes in the cAMP system associated with chronic opioid use. Currently, we are determining whether changes in the PI system or changes in the cAMP system associated with activation of metabotropic receptors are important for the development of tolerance and dependence with chronic opioid use.

Thus, the present study indicates that both NMDA and metabotropic glutamate receptors may be involved in the development of dependence with chronic morphine use. Both NMDA and metabotropic glutamate receptors are associated with changes in intracellular second messenger systems. It is therefore hypothesized that NMDA and metabotropic glutamate antagonists are effective because they prevent changes in second-messenger systems associated with chronic opioid use.

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Group II Metabotropic and α-Amino-3-hydroxy-5-methyl-4-isoxazole Propionate (AMPA)/Kainate Glutamate Receptors Regulate the Deficit in Brain Reward Function Associated with Nicotine Withdrawal in Rats

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ABSTRACT

This study investigated the role of ionotropic and metabotropic glutamate receptors in the deficits in brain reward function, as measured by elevations in intracranial self-stimulation (ICSS) reward thresholds, associated with nicotine withdrawal. The group II metabotropic glutamate (mGluII) receptor agonist LY314582 [a racemic mixture of LY354740 ([+]-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid])] (2.5–7.5 mg/kg) precipitated withdrawal-like elevations in ICSS thresholds, a sensitive measure of reward function, in nicotine-dependent but not control rats. LY314582 did not affect response latencies, a measure of performance in the ICSS paradigm. Bilateral microinfusion of LY314582 (10–100 ng/side) into the ventral tegmental area likewise precipitated dose-dependent threshold elevations in nicotine-dependent rats. Furthermore, a single injection of the mGluII receptor antagonist LY341495 (2S-2-amino-2-[1S,2S-2-carboxycyclopropan-1-yl]-3-[xanth-9-yl]propionic acid) (1 mg/kg) attenuated the threshold elevations observed in rats undergoing spontaneous nicotine withdrawal. mGluII receptors are primarily located on glutamatergic terminals throughout the mesocorticolimbic system, where they act as inhibitory autoreceptors. To investigate whether mGluII receptors contributed to nicotine withdrawal by decreasing glutamatergic transmission, we next examined whether direct blockade of postsynaptic glutamate receptors precipitated withdrawal-like reward deficits in nicotine-dependent rats. The α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline (NBQX; 0.01–1 mg/kg) precipitated withdrawal-like threshold elevations in nicotine-dependent but not control rats, whereas 6-methyl-2-[phenylethynyl]-pyridine (MPEP; 0.01–3 mg/kg) and dizocilpine (MK-801; 0.01–0.2 mg/kg), antagonists at metabotropic glutamate 5 and *N*-methyl-D-aspartate receptors, respectively, did not. Overall, these data demonstrate that mGluII receptors play an important role in the reward deficits associated with nicotine withdrawal. Furthermore, it is likely that mGluII receptors generate this reward deficit, at least in part, by decreasing glutamate transmission at AMPA/kainate receptors.

There is now compelling evidence that the aversive withdrawal syndrome observed during periods of nicotine abstinence contributes to the persistence of the tobacco habit in smokers (Hughes, 1992; Kenny and Markou, 2001). Nicotine withdrawal was shown to precipitate a deficit in brain reward function, as measured by elevations in intracranial self-stimulation (ICSS) reward thresholds, similar to that observed in rats undergoing withdrawal from other major drugs of abuse (Epping-Jordan et al., 1998). Moreover, avoidance and alleviation of this deficit in brain reward function has been proposed as a major motivational factor contributing to craving, relapse, and continued tobacco consumption in human smokers (Epping-Jordan et al., 1998; Kenny and Markou, 2001). In contrast to the intense investigations into the mechanisms by which acute nicotine produces its rewarding effects, little is known concerning the mechanisms mediating the reward deficits associated with nicotine withdrawal.

Most drugs of abuse have been shown to stimulate excitatory glutamatergic transmission throughout brain reward circuitries (Kalivas and Duffy, 1998; Wolf et al., 2000). Increases in glutamatergic transmission have been shown to play an important role in mediating the positive reinforcing actions of addictive drugs (Harris and Aston-Jones, 2003).

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ABBREVIATIONS: ICSS, intracranial self-stimulation; VTA, ventral tegmental area; mGluII, group II metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; mGlu5, metabotropic glutamate 5 receptors; MK-801, dizocilpine; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline; MPEP, 6-methyl-2-[phenylethynyl]-pyridine; ANOVA, analysis of variance.

Indeed, it is thought that nicotine elicits its rewarding actions, at least in part, by activating nicotinic acetylcholine receptors located on glutamate terminals in the ventral tegmental area (VTA), thereby potentiating excitatory glutamatergic transmission in this reward-relevant brain site and increasing mesoaccumbal dopamine transmission (Mansvelder and McGehee, 2000). Accordingly, blockade of glutamatergic transmission reduced nicotine's stimulatory action on mesoaccumbens dopamine transmission (Schilstrom et al., 1998) and attenuated the rewarding actions of nicotine and other drugs of abuse (Chiamulera et al., 2001; Laviolette and van der Kooy, 2003; Paterson et al., 2003).

It has been suggested that the neuroadaptations that occur during prolonged exposure to drugs of abuse, which give rise to the deficits in brain reward function associated with withdrawal, may reside in the same neural elements that mediate the acute rewarding actions of these drugs (Koob and Le Moal, 2001). Indeed, in contrast to nicotine's acute stimulatory effects, nicotine withdrawal attenuated mesoaccumbens dopamine transmission (Hildebrand et al., 1997), an action likely to contribute to the reward and motivational deficits associated with nicotine withdrawal (Kenny and Markou, 2001). Therefore, because increases in excitatory glutamatergic transmission are believed to play an important role in the reinforcing actions of acute nicotine, we hypothesized that withdrawal from nicotine is associated with decreased glutamatergic transmission in brain reward circuitries, which contributes to the reward deficits observed during withdrawal. To test this hypothesis, the effects of a group II metabotropic glutamate (mGluII) receptor agonist were examined in nicotine-treated and control rats. mGluII receptors, comprising of mGlu2 and mGlu3 receptors, are inhibitory autoreceptors located on glutamate terminals throughout the mesocorticolimbic system, where they act to decrease excitatory glutamatergic transmission (Bonci et al., 1997; Wigmore and Lacey, 1998). Because mGluII receptor agonists decrease glutamatergic transmission in brain reward circuitries (Manzoni and Williams, 1999), we predicted that activation of these receptors would precipitate ICSS threshold elevations in nicotine-dependent rats similar to those observed in rats during spontaneous nicotine withdrawal, whereas blockade of these receptors would reverse the threshold elevations associated with spontaneous nicotine withdrawal. To further investigate the role of glutamatergic transmission in nicotine withdrawal, we also examined whether direct blockade of glutamatergic transmission at postsynaptic NMDA, AMPA/kainate, and metabotropic glutamate 5 (mGlu5) receptors precipitated withdrawal-like ICSS threshold elevations in nicotine-dependent rats.

Materials and Methods

Animal Housing. Subjects were 149 male Wistar rats weighing 300 to 320 g at the start of each experiment. Rats were obtained from Charles River Laboratories (Raleigh, NC) and were housed in groups of two or three per cage, with food and water available ad libitum. Animals were maintained in a temperature-controlled vivarium under a 12-h light/dark cycle (lights off at 10:00 AM). Animals were tested during the dark portion of the light/dark cycle, except for the spontaneous nicotine withdrawal experiment when rats were tested at time points according to the experimental design. All animals were treated in accordance with the guidelines of the National Institutes of Health regarding the principles of animal care. Animal facilities and experimental protocols were in accordance with the Association for the Assessment and Accreditation of Laboratory Animal Care.

Drugs. (−)-Nicotine hydrogen tartrate salt ([−]-1-methyl-2-[3-pyridyl] pyrrolidine) and dizocilpine ([+]-MK-801 hydrogen maleate; [(5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo(*a*,*d*)cyclohepten-5,10-imine hydrogen maleate]) were purchased from Sigma-Aldrich (St. Louis, MO); LY341495 (2*S*-2-amino-2-[1*S*,2*S*-2-carboxycyclopropan-1-yl]-3-[xanth-9-yl]propionic acid) and NBQX disodium (2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(*f*)quinoxaline disodium) were purchased from Tocris Cookson (Ballwin, MO). LY314582 (the racemic mixture of LY354740 [(+)-2-aminobicyclo(3.1.0)hexane-2,6-dicarboxylic acid]) and 6-methyl-2-[phenylethynyl]-pyridine (MPEP) were synthesized by one of the coauthors (F. Gasparini). Drugs were prepared immediately before each administration. For systemic administration, all drugs were dissolved in sterile water and administered by intraperitoneal injection, in a volume of 1 ml/kg body weight, 30 min before the experimental session. For direct intra-VTA administration, LY314582 was dissolved in artificial cerebrospinal fluid of the following composition: 126.6 mM NaCl, 27.4 mM $NaHCO_3$, 2.4 mM KCl, 0.5 mM KH_2PO_4, 0.89 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.48 mM Na_2HPO_4 and 7.1 mM glucose, pH 7.4. Rats received intra-VTA injections immediately before the initiation of the experimental session. Unless otherwise stated, drug doses refer to the salt form.

Apparatus. Intracranial self-stimulation training and testing took place in 16 Plexiglas operant chambers (25 × 31 × 24 cm) (MED Associates, St. Albans, VT). The floors of the operant chambers were constructed of parallel aluminum rods spaced 1.25 cm apart. One wall contained a metal wheel manipulandum that required 0.2 N force to rotate it one-quarter of a turn. The wheel (5 cm in width) extended out of the wall ~3 cm. Each testing chamber was enclosed within a light- and sound-attenuated chamber (62 × 63 × 43 cm). Intracranial stimulation was delivered by constant current stimulators (Stimtech model 1200; San Diego Instruments, San Diego, CA). Subjects were connected to the stimulation circuit through flexible bipolar leads (Plastics One, Roanoke, VA) attached to gold-contact swivel commutators (model SL2C; Plastics One) mounted above the chamber. The stimulation parameters, data collection, and all test session functions were controlled by a microcomputer.

Placement of Electrodes and Cannulas. Rats were anesthetized by inhalation of 1 to 3% halothane in oxygen and positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA). The incisor bar was adjusted to 5 mm above the interaural line, and the skull exposed. Stainless steel bipolar electrodes (11 mm in length) were implanted into the posterior lateral hypothalamus (AP −0.5 mm from bregma; ML ±1.7 mm; DV 8.3 mm from dura), according to the atlas of Pellegrino et al. (1979). For the VTA infusion experiment, bilateral stainless steel guide cannulas (23-gauge, 14 mm in length) were implanted 3 mm above the VTA (AP −3.2 mm from bregma; ML ±1.7 mm; DV 5.3 mm from skull surface; angle of 10° from midline), at the same time that ICSS electrodes were implanted. Four indentations were made in the skull to accommodate screws that together with the application of dental acrylic, held the electrode and cannulas in place. Cannulas were kept patent using 14-mm-long stainless steel stylets (30-gauge). Animals were allowed to recover from surgery for at least 7 days before training in the ICSS paradigm.

Osmotic Mini-Pump Surgery. Rats were anesthetized by inhalation of 1 to 3% halothane in oxygen and prepared with Alzet osmotic mini-pumps [model 2ML4 (28 day); Alza, Palo Alto, CA] placed subcutaneously (back of the animal parallel to the spine). Pumps were filled with either sterile water or nicotine salt solution. The concentration of the nicotine salt solution was adjusted according to animal body weight, resulting in delivery of 9 mg/kg/day (3.16 mg/kg, free base). This dose of nicotine maintains stable plasma levels (~44 ng/ml) comparable with those obtained in human smokers consuming approximately 30 cigarettes per day (Benowitz, 1988). After mini-pump implantation (or removal), the surgical wound was

closed with 9-mm stainless steel wound clips (BD Biosciences Primary Care Diagnostics, Sparks, MD) and treated with topical antibiotic (Bacitracin) ointment.

ICSS Reward Threshold Procedure. Animals were trained to respond according to a modification of the discrete-trial current-threshold procedure of Kornetsky and Esposito (1979). Briefly, a trial was initiated by the delivery of a noncontingent electrical stimulus. This electrical reinforcer had a train duration of 500 ms and consisted of 0.1-ms rectangular cathodal pulses that were delivered at a frequency of 50 to 100 Hz. The frequency of the stimulation was selected for individual animals so that current-intensity thresholds of each subject were within 85 to 160 μA, and thus allowed both threshold elevations and lowerings to be detected. This frequency was held constant throughout the experiment. A one-quarter turn of the wheel manipulandum within 7.5 s of the delivery of the noncontingent electrical stimulation resulted in the delivery of an electrical stimulus identical in all parameters to the noncontingent stimulus that initiated the trial. After a variable intertrial interval (7.5–12.5 s, average of 10 s), another trial was initiated with the delivery of a noncontingent electrical stimulus. Failure to respond to the noncontingent stimulus within 7.5 s resulted in the onset of the intertrial interval. Responding during the intertrial interval delayed the onset of the next trial by 12.5 s. Current levels were varied in alternating descending and ascending series. A set of three trials was presented for each current intensity. Current intensities were altered in 5-μA steps. In each testing session, four alternating descending and ascending series were presented. The threshold for each series was defined as the midpoint between two consecutive current intensities that yielded "positive scores" (animals responded for at least two of the three trials) and two consecutive current intensities that yielded "negative scores" (animals did not respond for two or more of the three trials). The overall threshold of the session was defined as the mean of the thresholds for the four individual series. Each testing session was ~30 min in duration. The time between the onset of the noncontingent stimulus and a positive response was recorded as the response latency. The response latency for each test session was defined as the mean response latency of all trials during which a positive response occurred. After establishment of stable ICSS reward thresholds, rats were tested in the ICSS procedure once daily except for the spontaneous nicotine withdrawal experiment when rats were tested at time points according to the experimental design.

Intracerebral Injection Procedure. All injections were administered bilaterally in a volume of 0.5 μl/side given over 66 s through 17-mm injectors. The injectors were connected to calibrated polyethylene-10 tubing preloaded with drug solution and protruded 3 mm below the ends of the cannulas into the VTA. After infusion, the injectors were kept in place for an additional 60 s to allow for drug diffusion and to minimize diffusion along the injection tract when pulling out the injector. Injectors were then removed and replaced with 14-mm wire stylets, and the animals were placed directly into the ICSS testing apparatus. Injections were made using a microinfusion pump (model 975; Harvard Apparatus Inc., Holliston, MA).

Systemic Drug Administration Experiments. These experiments investigated whether nicotine withdrawal, as measured by elevations in ICSS thresholds, could be precipitated in nicotine-treated rats by systemic administration of an agonist at mGluII receptors (LY314582), or antagonists at mGlu5 (MPEP), NMDA (dizocilpine), or AMPA/kainate (NBQX) glutamate receptors. For each drug tested, rats were trained in the ICSS paradigm until stable baseline responding was achieved, defined as ≤10% variation in thresholds for three consecutive days and requiring approximately 14 days of daily testing. In each case, drug-naïve rats were then assigned to two separate groups such that there was no difference in mean baseline ICSS thresholds or body weight between groups. One group was then prepared with subcutaneous osmotic mini-pumps delivering vehicle and the second group with mini-pumps delivering 9 mg/kg/day nicotine hydrogen tartrate (3.16 mg/kg/day nicotine free base). There was a minimum 7-day interval after mini-pump implantation, during which ICSS reward thresholds continued to be measured daily, before the effect of any systemically administered drug on reward thresholds was evaluated. This time period was sufficient to produce robust elevations in thresholds in nicotine-treated but not vehicle-treated rats upon abrupt removal of mini-pumps (i.e., spontaneous withdrawal) or administration of nicotinic receptor antagonists (i.e., precipitated withdrawal; Epping-Jordan et al., 1998). Separate groups of nicotine-treated rats and their corresponding nicotine-naive control group were then injected intraperitoneally with the mGluII receptor agonist LY314582 (0, 2.5, 0.5, and 7.5 mg/kg; n = 9 nicotine, n = 11 control), the mGlu5 receptor antagonist MPEP (0, 0.01, 0.05, and 0.1 mg/kg; n = 8 nicotine, n = 7 vehicle or 0, 0.5, 1, 2, and 3 mg/kg; n = 13 nicotine, n = 13 vehicle), the NMDA receptor antagonist dizocilpine (0, 0.01, 0.05, 0.1, 0.175, and 0.2 mg/kg; n = 10 nicotine, n = 9 control), or the AMPA/kainate receptor antagonist NBQX (0, 0.01, 0.025, 0.05, 0.075, 0.1, 0.5, and 1 mg/kg; n = 10 nicotine, n = 12 control) according to within-subjects Latin-square designs and ICSS thresholds were evaluated 30 min later. A minimum of 48 h was allowed between each injection in the Latin-square design, during which ICSS thresholds continued to be measured daily, to ensure that ICSS thresholds returned to baseline levels before the next drug administration. The doses of LY314582 and MPEP were chosen based on a previous study demonstrating that ≥10 mg/kg LY314582 and ≥3 mg/kg MPEP elevated ICSS thresholds in drug-naïve rats (Harrison et al., 2002). For the potential demonstration of statistical interaction effects, it was important to include doses of the test drugs that did not alter thresholds under baseline conditions.

Intraventral Tegmental Area Administration Experiment. After stable baseline ICSS responding was achieved (≤10% variation in threshold for three consecutive days), rats (n = 15) with bilateral cannulas directed toward the VTA were allocated to two groups such that there were no differences in mean baseline reward thresholds or body weight between groups. One group was then prepared with subcutaneous osmotic mini-pumps delivering vehicle and a second group with mini-pumps delivering nicotine (3.16 mg/kg/day nicotine free-base). Animals again were tested in the ICSS paradigm each day for 7 days before drug treatment. Both groups of rats were then injected directly into the VTA, as described above, with LY314582 (0, 10, 50, and 100 ng/side; n = 7 nicotine, n = 8 control) according to a within-subjects Latin square design, and ICSS reward thresholds were evaluated immediately postinjection. There was a minimum 48-h interval between each injection, during which ICSS thresholds continued to be measured, to allow thresholds to return to baseline levels before further drug tests. At the conclusion of the experiment, all animals were anesthetized and their brains removed and immediately placed on ice. The brains were cut in 50-μm sections, and placements of the injectors and the electrodes were examined (Fig. 1 for histological verification of injection sites). Only those rats with injection tips located within the VTA were included in statistical analyses.

Spontaneous Nicotine Withdrawal Experiment. Osmotic mini-pumps were surgically removed from nicotine-treated rats (n = 15) (defined as rats having been prepared with mini-pumps delivering 3.16 mg/kg/day nicotine free-base for at least 7 days) or corresponding control rats (n = 17; rats prepared with vehicle-containing mini-pumps). All rats were then tested in the ICSS procedure at 12, 18, 24, 36, 48, and 72 h after the removal of osmotic mini-pumps. These time points were chosen based on the time course of threshold elevations previously observed during spontaneous nicotine withdrawal after removal of nicotine-delivering osmotic mini-pumps (Harrison et al., 2001). Based on the ICSS reward thresholds obtained at the 12-h time point, nicotine-withdrawing rats were allocated to two groups such that there was no difference in the magnitude of reward threshold elevations between each group (117.67 ± 3.1%, n = 8; 119.93 ± 3.5%, n = 7). Similarly, control rats were allocated to two groups such that there was no difference in mean reward thresholds between these groups (106.45 ± 5.2%, n = 7; 103.63 ± 3.6%, n = 10). Thirty min before being tested at the 18-h

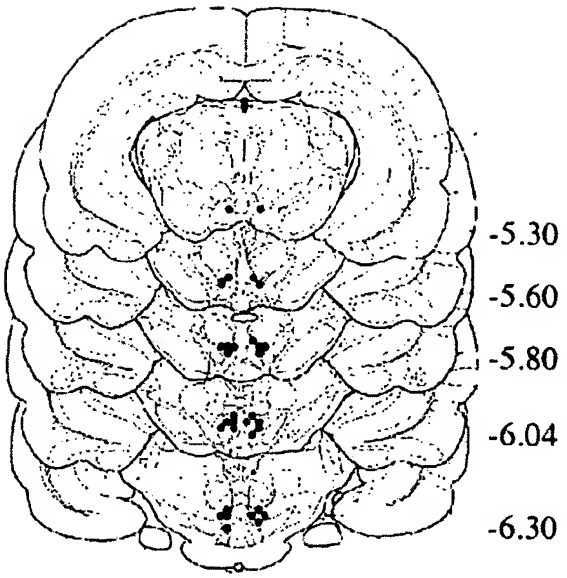

Fig. 1. Diagrammatic representation of coronal sections from the rat brain showing histological reconstruction of the injection sites in the ventral tegmental area. Black circles indicate locations of injector tips located inside the VTA (5.30–6.30 mm posterior to bregma, according to the atlas of Paxinos and Watson, 1986), and included in statistical analysis. Data from rats with injection sites located outside the VTA were removed from the analyses.

time point, one group of nicotine withdrawing and one group of control rats were injected with LY341495 (1 mg/kg); the remaining rats were injected with vehicle.

Statistical Analyses. Mean raw thresholds and response latencies (± S.E.M.) are presented for each experiment in the results section. For all experiments, except the spontaneous nicotine withdrawal experiment, percentage of change from baseline reward threshold was calculated by expressing the drug-influenced raw threshold scores as a percentage of the previous day's threshold (i.e., a drug-free baseline threshold). These percentages of baseline scores were subjected to two-factor repeated-measures analyses of variance (ANOVA), with treatment drug dose as the within-subjects factor and pump content (nicotine or control) as the between-subjects factor. For the spontaneous nicotine withdrawal experiment, percentage change from baseline reward threshold was calculated by expressing the threshold scores obtained at each time point during withdrawal as a percentage of thresholds for each rat on the day immediately before mini-pump removal. These percentages of baseline scores were subjected to three-factor repeated measures ANOVA. The within-subjects factor was the time after mini-pump removal, and the two between-subjects factors were pump content (nicotine or vehicle) and acute drug treatment (LY314582 or vehicle). For all experiments, response latency data were analyzed in the same manner as the threshold data. After statistically significant effects in the ANOVAs, post hoc comparisons among means were conducted with the Fisher's least significant difference test. The level of significance was set at 0.05.

Results

Systemic Administration of the mGluII Receptor Agonist LY314582 Precipitated Elevations in ICSS Thresholds in Nicotine-Treated but Not Control Rats. Mean (±S.E.M.) raw reward thresholds before treatment with the mGluII receptor agonist LY314582 for control and nicotine-treated rats were 98.9 ± 11.6 and 96.3 ± 12.3 μA, respectively. Mean (±S.E.M.) raw response latencies for control and nicotine-treated rats were 3.38 ± 0.2 and 3.21 ± 0.14 μA, respectively. Intraperitoneal administration of LY314582 (2.5–7.5 mg/kg) elevated ICSS reward thresholds in nicotine-treated but not control rats. This effect was reflected in a statistically significant effect of group [$F_{(1,18)}$ = 7.43, $p < 0.05$], a significant effect of dose [$F_{(3,54)}$ = 5.02, $p < 0.005$], and a significant group × dose interaction [$F_{(3,54)}$ = 2.79, $p < 0.05$]. Post hoc analysis revealed that the highest dose of LY314582 (7.5 mg/kg) elevated reward thresholds in nicotine-treated rats compared with vehicle treatment ($p < 0.01$) and compared with control rats tested with the same dose ($p < 0.01$) (Fig. 2). In contrast to its effects on reward thresholds, LY314582 had no effect on response latencies in nicotine-treated or control rats [$F_{(3,54)}$ = 0.59, N.S.] at any dose tested (data not shown).

Ventral Tegmental Area Administration of the mGluII Receptor Agonist LY314582 Precipitated Elevations in ICSS Reward Thresholds in Nicotine-Treated but Not Control Rats. Mean (±S.E.M.) raw reward thresholds before intra-VTA administration of LY314582 for control and nicotine-treated rats were 115.3 ± 12.2 and 113.5 ± 19.0 μA, respectively. Mean (±S.E.M.) raw response latencies for control and nicotine-treated rats were 3.55 ± 0.29 and 3.15 ± 0.13 s, respectively. Bilateral microinfusion of LY314582 (10–100 ng/side) directly into the VTA significantly elevated reward thresholds in nicotine-treated but not control rats (Fig. 3). Again, there were significant effects of group [$F_{(1,13)}$ = 4.81, $p < 0.05$], dose [$F_{(3,39)}$ = 4.77, $p < 0.01$], and a significant group × dose interaction [$F_{(3,39)}$ = 3.82, $p < 0.05$]. Post hoc analyses revealed that doses of 50 and 100 ng/side LY314582 were sufficient to elevate reward thresholds in nicotine-treated rats without affecting thresholds in control rats. LY314582 had no effect on response latencies [$F_{(3,39)}$ = 1.94, N.S.] in nicotine-treated or control rats after VTA administration (data not shown).

The mGluII Receptor Antagonist LY341495 Attenuated the Elevations in ICSS Thresholds Associated with Spontaneous Nicotine Withdrawal. Mean (±S.E.M.) raw reward thresholds prior to mini-pump re-

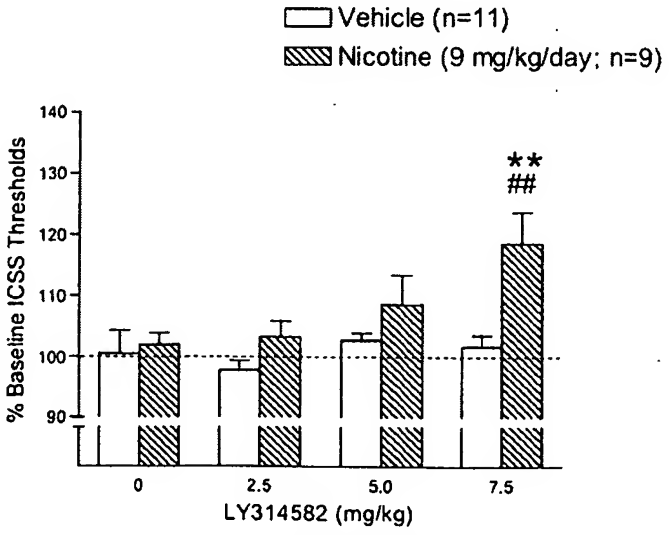

Fig. 2. Effects of LY314582 on ICSS thresholds in nicotine-treated and control rats. Data are expressed as mean (±S.E.M.) percentage change from baseline thresholds. **, $p < 0.01$, different from nicotine-treated rats after vehicle injection. ##, $p < 0.01$, different from control rats after injection with same dose of LY314582.

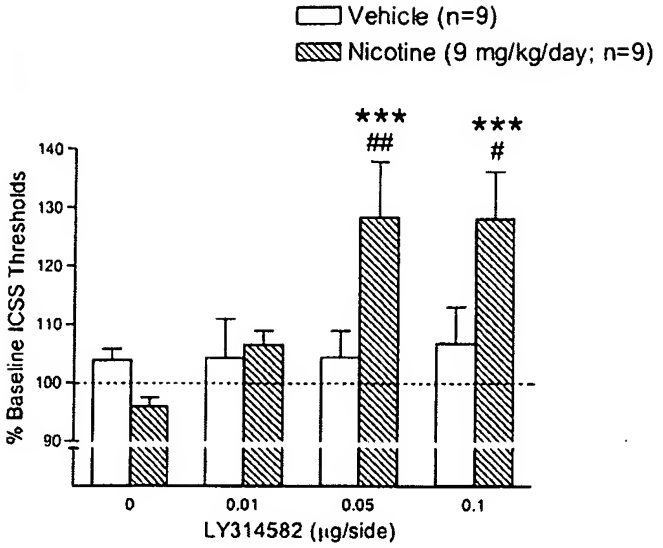

Fig. 3. Effects of intraventral tegmental area LY314582 on ICSS thresholds in nicotine-treated and control rats. Data are expressed as mean (±S.E.M.) percentage change from baseline thresholds. ***, $p < 0.001$, different from nicotine-treated rats after vehicle injection. ##, $p < 0.01$; #, $p < 0.05$, different from control rats after injection with same dose of LY314582.

moval for control and nicotine-treated rats were 105.9 ± 7.8 and 105.9 ± 11.0 μA, respectively. Mean (±S.E.M.) raw response latencies for control and nicotine-treated rats were 3.38 ± 0.1 and 3.36 ± 0.13 μA, respectively. Withdrawal from chronic nicotine treatment produced robust ICSS threshold elevations compared with control rats [$F_{(1,27)} = 15.3$, $p < 0.001$] (Fig. 4A). Analysis of the significant group × dose × time interaction [$F_{(5,135)} = 3.3$, $p < 0.02$] revealed the following. Nicotine-treated rats injected with vehicle demonstrated robust reward threshold elevations that reached a peak 24 h after mini-pump removal (Fig. 4A). However, administration of LY341495 30 min before the 18-h time point significantly attenuated the elevations in reward thresholds in nicotine-withdrawing rats ($p < 0.001$) (Fig. 4A), without affecting thresholds in control rats (Fig. 4B). LY341495 had no effect on response latencies at any time point after injection [$F_{(1,27)} = 0.43$, N.S.] in nicotine-treated or control rats (data not shown).

The NMDA Receptor Antagonist Dizocilpine Lowered ICSS Thresholds Similarly in Nicotine-Treated and Control Rats. Mean (±S.E.M.) raw reward thresholds before treatment with the NMDA receptor antagonist dizocilpine for control and nicotine-treated rats were 88.9 ± 9.1 and 86.9 ± 3.2 μA, respectively. Mean (±S.E.M.) raw response latencies for control and nicotine-treated rats were 3.32 ± 0.06 and 3.10 ± 0.06 μA, respectively. As can be seen in Fig. 5A, dizocilpine (MK-801; 0.01–0.2 mg/kg) lowered ICSS reward thresholds in nicotine-treated and control rats [$F_{(6,66)} = 7.5$, $p < 0.0001$], and there was no group × dose interaction [$F_{(6,66)} = 1.2$, N.S.]. Doses of dizocilpine ≥0.2 mg/kg caused disruption in performance in the ICSS paradigm in both groups such that rats no longer responded for self-stimulation, and therefore doses higher than 0.2 mg/kg were not tested. Furthermore, dizocilpine did not precipitate withdrawal-like elevations in reward thresholds in nicotine-treated rats at any dose tested. Dizocilpine significantly increased response latencies [$F_{(6,72)} = 2.9$, $p < 0.05$]. Post hoc analysis demonstrated that as the dose of dizocilpine increased, so too did response latency, particularly in control rats, suggesting that performance was increasingly impaired at higher doses of dizocilpine (Fig. 5B).

The mGlu5 Receptor Antagonist MPEP Elevated ICSS Thresholds Similarly in Nicotine-Treated and Control Rats. Mean (±S.E.M.) raw reward thresholds before treatment with low doses of the mGlu5 receptor antagonist MPEP for control and nicotine-treated rats were 118.9 ± 9.3 and 98.4 ± 8.9 μA, respectively. Mean (±S.E.M.) raw response latencies for the low-dose MPEP experiment for control and nicotine-treated rats were 3.34 ± 0.09 and 3.43 ±

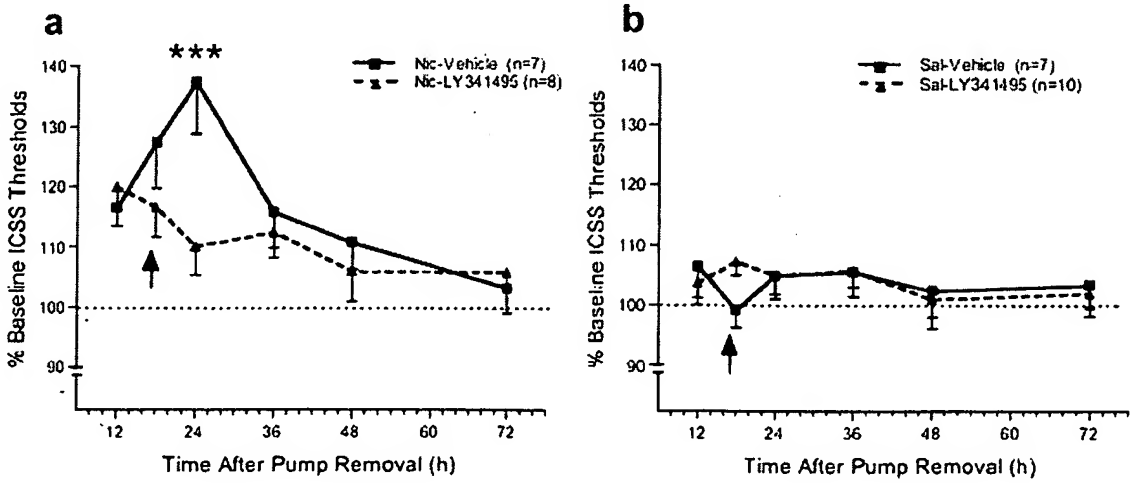

Fig. 4. Effects of LY341495 on the elevations in ICSS thresholds in rats undergoing spontaneous nicotine withdrawal. A, data are expressed as mean (±S.E.M.) percentage change from baseline thresholds in rats undergoing nicotine withdrawal. B, data are expressed as mean (±S.E.M.) percentage change from baseline thresholds in saline-treated control rats. ICSS thresholds were tested 12, 18, 24, 36, 48, and 72 h after surgical removal of osmotic mini-pumps delivering nicotine (Nic; A) or saline (Sal; B). Rats received a single injection of LY341495 (1 mg/kg) or vehicle (Veh) 30 min before the 18-h time point (indicated by black arrows). Solid line represents nicotine-withdrawing (A) or nicotine-naive (B) rats treated with vehicle 30 min before testing at the 18-h time point. Dashed line represents nicotine-withdrawing (A) or nicotine-naive (B) rats treated with LY341495 (1 mg/kg) 30 min before testing at the 18-h time point. ***, $p < 0.001$, different from rats undergoing nicotine withdrawal treated with vehicle 30 min before the 18-h time point.

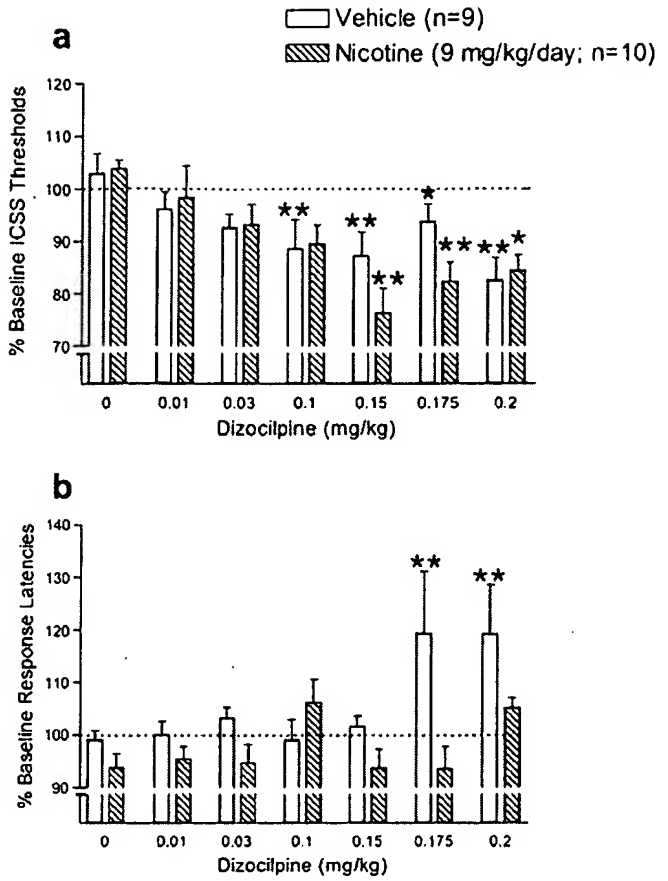

Fig. 5. Effects of dizocilpine (MK-801) on ICSS thresholds and response latencies in nicotine-treated and control rats. A, data are expressed as mean (±S.E.M.) percentage change from baseline thresholds. B, data are expressed as mean (±S.E.M.) percentage change from baseline response latencies. *, $p < 0.05$; **, $p < 0.01$, different from corresponding vehicle- or nicotine-treated rats after vehicle injection.

0.12 s, respectively. Mean (±S.E.M.) raw reward thresholds before treatment with high doses of MPEP for control and nicotine-treated rats were 112.9 ± 8.9 and 109.5 ± 8.5 μA, respectively. Mean (±S.E.M.) raw response latencies for the high-dose MPEP experiment for control and nicotine-treated rats were 3.27 ± 0.19 and 3.14 ± 0.07 s, respectively. Low doses of MPEP (0.01–0.1 mg/kg) did not affect ICSS reward thresholds [$F_{(3,39)} = 2.3$, N.S.] or response latencies [$F_{(3,39)} = 0.4$, N.S.] in nicotine-treated or control rats (data not shown). Higher doses of MPEP (0.5–3 mg/kg) elevated ICSS thresholds in nicotine-treated and control rats [$F_{(4,96)} = 8.4$, $p < 0.0001$] (Fig. 6). However, MPEP elevated ICSS thresholds in both groups of rats by a similar magnitude (Fig. 6), and there was no group × dose interaction [$F_{(4,96)} = 0.7$, N.S.]. MPEP (0.5–3 mg/kg) had no effect on response latencies [$F_{(4,96)} = 1.4$, N.S.] in either group (data not shown).

The AMPA/Kainate Receptor Antagonist NBQX Precipitated Elevations in ICSS Thresholds in Nicotine-Treated but Not Control Rats. Mean (±S.E.M.) raw reward thresholds prior to treatment with the AMPA/kainate receptor antagonist for control and nicotine-treated rats were 98.9 ± 10.0 and 98.5 ± 11.8 μA, respectively. Mean (±S.E.M.) raw response latencies for control and nicotine-treated rats were 3.21 ± 0.09 and 3.36 ± 0.15 μA, respectively. NBQX (0.01–1 mg/kg) significantly altered ICSS

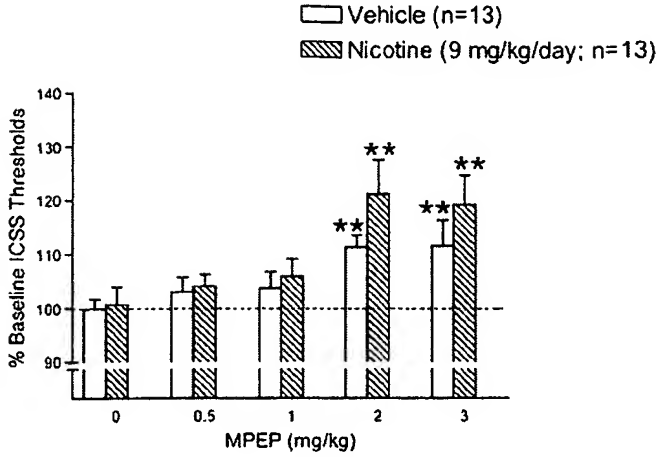

Fig. 6. Effects of MPEP on ICSS thresholds in nicotine-treated and control rats. Data are expressed as mean (±S.E.M.) percentage change from baseline thresholds. **, $p < 0.01$, different from corresponding vehicle- or nicotine-treated rats after vehicle injection.

thresholds in nicotine-treated but not control rats (Fig. 7). This effect was reflected in a statistically significant effect of group [$F_{(1,20)} = 10.82$, $p < 0.005$], a significant effect of dose [$F_{(7,140)} = 2.8$, $p < 0.01$], and a significant group × dose interaction [$F_{(7,140)} = 2.11$, $p < 0.05$]. Post hoc analysis revealed a bimodal action of NBQX on ICSS thresholds in nicotine-treated rats. Low doses of NBQX (0.025–0.1 mg/kg) elevated thresholds in nicotine-treated rats, whereas higher doses of NBQX (0.5–1 mg/kg) were less effective and did not significantly elevate thresholds compared with vehicle treatment (Fig. 7). NBQX had no effect on response latencies in nicotine-treated or control rats at any dose tested [$F_{(7,140)} = 0.31$, N.S.] (data not shown).

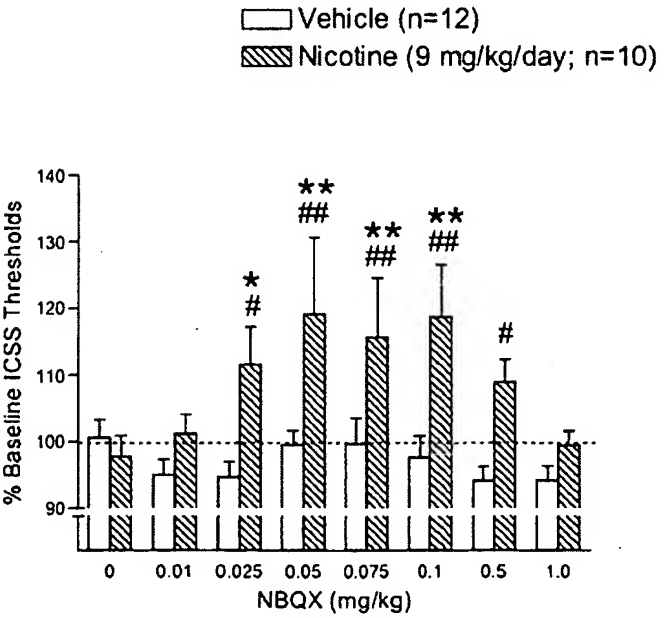

Fig. 7. Effects of NBQX on ICSS thresholds in nicotine-treated and control rats. Data are expressed as mean (±S.E.M.) percentage change from baseline thresholds. *, $p < 0.05$; **, $p < 0.01$, different from nicotine-treated rats after vehicle injection. #, $p < 0.05$; ##, $p < 0.01$, different from control rats after injection with same dose of NBQX.

Discussion

Nicotine withdrawal precipitates an aversive abstinence syndrome in human smokers hypothesized to provide an important source of motivation contributing to the persistence of the smoking habit and relapse during abstinence (Kenny and Markou, 2001). The present data strongly suggest a role for group II metabotropic glutamate receptors in generating the reward deficits associated with nicotine withdrawal by demonstrating that activation of mGluII receptors precipitated ICSS threshold elevations in nicotine-dependent rats similar to those observed during spontaneous nicotine withdrawal. Furthermore, activation of mGluII receptors in the VTA also elevated thresholds in nicotine-dependent rats, providing further support for an important role of the VTA in mediating the actions of nicotine on reward pathways. Consistent with the above-mentioned information, blockade of mGluII receptors attenuated the reward deficits in rats undergoing spontaneous nicotine withdrawal. Previously, the mGluII receptor agonist LY354740 was shown to attenuate the increased auditory startle observed during spontaneous nicotine withdrawal (Helton et al., 1997). One possible explanation for these observations is that mGluII receptors located in different brain sites may differentially regulate various aspects of nicotine withdrawal.

Previously, the mGluII receptor agonist LY314582, which we found here to elevate reward thresholds in nicotine-dependent rats at doses ≤7.5 mg/kg, was shown to elevate ICSS thresholds in control rats at doses ≥10 mg/kg (Harrison et al., 2002). Therefore, the present observation that "subthreshold" doses of LY314582 precipitated withdrawal-like threshold elevations in nicotine-dependent but not control rats suggests that negative regulation of brain reward function by mGluII receptors was increased by prolonged nicotine treatment. One mechanism through which nicotine elicits its reinforcing effects is by increasing glutamatergic transmission in the VTA, thereby potentiating mesoaccumbens dopamine transmission (Schilstrom et al., 1998). Because mGluII receptors located in the VTA are presynaptic autoreceptors that decrease glutamate transmission (Bonci et al., 1997; Wigmore and Lacey, 1998), it is likely that the increased mGluII receptor sensitivity in the VTA observed in nicotine-treated rats occurred in response to prolonged activation of excitatory glutamate transmission by nicotine in this brain site, perhaps to counter this effect. Thus, during nicotine withdrawal when the stimulatory effects of nicotine on excitatory glutamate transmission were no longer present, increased mGluII receptor function would be expected to decrease glutamate transmission and thereby decrease the activity of this brain reward substrate. Recent electrophysiological studies are consistent with this hypothesis. For instance, chronic opiate treatment increased the inhibitory effects of mGluII receptor agonists on excitatory glutamate currents in VTA dopamine neurons (Manzoni and Williams, 1999), and in nucleus accumbens neurons (Martin et al., 1999). Nevertheless, it is possible mGluII receptors are also located on nonglutamatergic terminals (e.g., serotonergic and cholinergic neurons) and that activation of mGluII receptors precipitated nicotine withdrawal by decreasing the release of neurotransmitters other than glutamate. Indeed, nicotine withdrawal-induced threshold elevations were attenuated by coadministration of fluoxetine, a selective serotonin reuptake inhibitor, and 4-(2′-methoxyphenyl)-1-[2′(*N*-[2′-pyridinyl]-*p*-iodo-benzamido)ethyl]piperazine (P-MPPI), a serotonin-1A receptor antagonist, suggesting that decreased serotonergic transmission also contributes to the reward deficits associated with nicotine withdrawal (Harrison et al., 2001).

To further investigate a potential role of decreased glutamatergic transmission in the reward deficits associated with nicotine withdrawal, we examined whether antagonists at postsynaptic glutamate receptors precipitated withdrawal-like threshold elevations in nicotine-dependent rats similar to activation of mGluII receptors. At low doses the AMPA/kainate receptor antagonist NBQX precipitated threshold elevations in nicotine-treated but not controls rats. Under "normal" baseline conditions, AMPA receptors are the primary regulators of excitatory glutamate transmission throughout the mesoaccumbens reward pathway (Pennartz et al., 1990). Furthermore, AMPA receptor overexpression in the VTA increased, whereas AMPA receptor blockade decreased, the rewarding actions of drugs of abuse (Carlezon et al., 1997; Xi and Stein, 2002). These observations suggest that AMPA receptors positively modulate brain reward function. Conversely, AMPA receptor antagonists elicit an intrinsic rewarding action after VTA administration (David et al., 1998), suggesting that AMPA receptors may also negatively regulate brain reward function under baseline conditions. Indeed, AMPA receptors are located on dopamine and GABAergic neurons in the VTA (Wang and French, 1993, 1995), where they modulate mesoaccumbens dopamine transmission in an opposite manner. Therefore, it is possible that NBQX had no effects in control rats because it simultaneously blocked populations of AMPA/kainate receptors that positively and negatively regulate reward function. However, the sensitivity of nicotine-treated rats to NBQX suggests a scenario in which the development of nicotine dependence led to compensatory decreases in the number and/or function of those AMPA/kainate receptors that positively regulate brain reward function, perhaps to counter the prolonged stimulatory effects of nicotine on reward pathways. Consistent with this hypothesis, prolonged nicotine exposure decreased AMPA receptor immunoreactivity in the VTA and nucleus accumbens (Lee et al., 2002). Alternatively, it is possible that a "silent" population of AMPA/kainate receptors was recruited during prolonged nicotine exposure (Isaac et al., 1995), resulting in increased regulation of reward circuitries by AMPA/kainate receptors. Regardless of the mechanism, these data suggest that decreased glutamatergic transmission at AMPA/kainate receptors contributes to the threshold elevations observed in nicotine withdrawing rats.

There is considerable evidence that NMDA receptors play an important role in mediating the stimulatory effects of nicotine on mesoaccumbens dopamine transmission (Grillner and Svensson, 2000). Therefore, it might have been expected that prolonged nicotine treatment may have resulted in adaptations in the function/number of NMDA receptors such that their blockade precipitated withdrawal-like threshold elevations in nicotine-dependent rats but not controls similar to AMPA/Kainate receptor blockade. Nevertheless, this did not seem to be the case. Similar to previous reports (Carlezon and Wise, 1993), NMDA receptor blockade lowered thresholds in nicotine-dependent and control rats, indicating a rewarding action. At no dose tested did the NMDA receptor antagonist dizocilpine elevate thresholds in either nicotine-

treated or control rats. Interestingly, dizocilpine tended to lower thresholds by a greater magnitude in nicotine-treated rats, suggesting they were slightly more sensitive to dizocilpine's reward-facilitating effects. Furthermore, higher doses of dizocilpine elevated response latencies in control but not nicotine-dependent rats, suggesting that prolonged nicotine treatment attenuated the performance-disrupting effects of dizocilpine. Nevertheless, based on the present data it is unlikely that decreased glutamatergic transmission at NMDA receptors contributes to the threshold elevations associated with nicotine withdrawal.

Recently, mGlu5 receptors, which are primarily located postsynaptically throughout the mesocorticolimbic system (Wigmore and Lacey, 1998), were shown to block the reinforcing effects of drugs of abuse, including nicotine (Chiamulera et al., 2001; Paterson et al., 2003). Therefore, we also investigated the role of mGlu5 receptors in nicotine withdrawal. At low doses, the mGlu5 receptor antagonist MPEP had no effect on ICSS thresholds, whereas higher doses elevated thresholds in nicotine-dependent and control rats (consistent with Harrison et al., 2002). Interestingly, MPEP tended to elevate thresholds by a greater magnitude in nicotine-dependent rats compared with control. However, because no dose of MPEP differentially elevated thresholds in nicotine-treated rats without also elevating thresholds similarly in control rats, these data indicate that mGlu5 receptors regulate baseline brain reward function in control and nicotine-treated rats, but are probably not involved in the threshold elevations associated with nicotine withdrawal.

Perhaps the most parsimonious explanation of the present observations is that prolonged, continuous nicotine exposure increased mGluII receptor function, and decreased AMPA/kainate-mediated glutamate transmission in reward circuitries, which contributed to the reward deficits observed during nicotine withdrawal. In contrast, recent investigations demonstrated that repeated, intermittent exposure to psychostimulants decreased mGluII function, and increased AMPA receptor transmission in reward circuitries (Giorgetti et al., 2001; Xi et al., 2002). Thus, it is possible that chronic nicotine and psychostimulant administration induce different alterations in glutamatergic transmission. Alternatively, this apparent discrepancy may be explained by the fact that the long-term behavioral effects of drugs of abuse are related to the dosing administration regimen (i.e., continuous or intermittent). Specifically, repeated intermittent exposure to addictive drugs can result in a progressive augmentation or "sensitization" in their behavioral effects (Pierce and Kalivas, 1997; Wolf, 1998). Conversely, more continuous exposure similar to that used in the present study, and similar to the pattern of prolonged nicotine exposure observed in smokers, engages counteradaptive "opponent processes" that decrease the acute behavioral effects of addictive drugs (i.e., "tolerance"), and leads to the expression of an aversive withdrawal syndrome upon cessation (Koob and Le Moal, 2001). It has been proposed that sensitization may be important in the early stages of drug addiction, when intake is intermittent, whereas tolerance and withdrawal may be more important in later stages of drug dependence, as drug intake progressively increases (Koob and Le Moal, 2001; Kenny et al., 2003). Based on the above-mentioned information, it is an interesting possibility that an initial increase, followed by a prolonged decrease in glutamatergic transmission, mediated by mGluII and AMPA/kainate receptors, may be involved in the initiation and maintenance of the drug-taking habit, respectively. Thus, it will be of interest to investigate whether other major drugs of abuse also increase the regulation of brain reward function by mGluII receptors.

In conclusion, the present data suggest that mGluII receptors play an important role in generating the reward deficits associated with nicotine withdrawal. Furthermore, it is likely that mGluII receptors generated these deficits, at least in part, by decreasing glutamate transmission at AMPA/kainate receptors. Thus, because the reward deficits associated with drug withdrawal are thought to play such a crucial role in drug addiction (Ahmed et al., 2002; Kenny et al., 2003), these data suggest that mGluII and AMPA/kainate glutamate receptors may prove to be useful therapeutic targets for the treatment of nicotine addiction.

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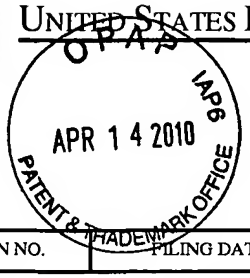

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/527,525	10/14/2005	Athina Markou	TSRI 897.1	3218

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EXAMINER	
CARTER, KENDRA D	
ART UNIT	**PAPER NUMBER**
1627	

MAIL DATE	DELIVERY MODE
12/15/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Ref. 17 (attached to Appeal Brief; USSN 10/527,525)

Office Action Summary

Application No.	Applicant(s)	
10/527,525	MARKOU ET AL.	
Examiner	**Art Unit**	
KENDRA D. CARTER	1627	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *31 August 2009*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-3,6,7,9,16,17,19,27,28 and 32* is/are pending in the application.

4a) Of the above claim(s) *10,14,15,17 and 19* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-3,6,7,9,16,27,28 and 32* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *6/22/09*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

The Examiner acknowledges the applicant's remarks of August 31, 2009 made to the office action filed June 24, 2009. Claims 1-3, 6, 7, 9, 10, 14-17, 19, 27, 28 and 32 are pending. Claims 4, 5, 8, 9, 11-13, 18, 20-26, 29-31 and 33 are canceled. Claims 10, 14, 15, 17 and 19 are withdrawn. Claims 1-3, 6, 7, 19 and 27 are amended.

For the reasons in the previous office action and below, the Applicant's arguments of the following 35 U.S.C. 103(a) rejections were found not persuasive, thus the rejection is upheld: 1) claims 1-8, 16, 20, 21 and 29 as being unpatentable over Adam et al. in view of Corsi et al. or Chimulera et al.; and 2) claims 9, 22, 27, 28, 32 and 33 as being unpatentable over Chiamulera et al. in view of Adam et al. as applied to claims 1-8, 16, 18, 20, 21 and 29 above. Both rejections are modified in light of the cancelled and amended claims.

Due to the amendments to the claims the modified 35 U.S.C. 103(a) rejections are made below. Applicant's arguments are addressed below.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

The factual inquiries set forth in *Graham* **v.** *John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103(a) are summarized as follows:

1. Determining the scope and contents of the prior art.
2. Ascertaining the differences between the prior art and the claims at issue.
3. Resolving the level of ordinary skill in the pertinent art.
4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

(1) Claims 1-3, 6, 7 and 16 are rejected under 35 U.S.C. 103(a) as being unpatentable over Adam et al. (US 6407,094 B1) in view of Corsi et al. (US 2003/0195139 A1) or Chiamulera et al. (Nature Neuroscience, 2001, vol. 4(9), pp. 873-874).

Adam et al. teaches compounds that act as Group II (i.e. mGluR 2 and 3) metaboltropic glutamate receptor antagonist (see column 16, lines 47 and 48) and treat conditions such as nicotine addiction, and opiate addiction (see column 1, lines 54-56

and column 3, lines 20-24; addresses claims 1-3, 6, 7 and 16). The antagonist can be in their pharmaceutically acceptable salts (see column 3, line 4).

Adam et al. does not teach an antagonist which modulated metabotropic glutamate receptor 5, or its administration in combination with the antagonist of Adam et al.

Corsi et al. teaches a method of treating substance dependence, wherein the substance is nicotine, opiate, cocaine, amphetamine, benzodiazepine and ethanol, comprising administering a therapeutically effective amount of an antagonist of mGluR5 (see claims 21-23; addresses claims 1-3, 6, 7 and 16). The compounds can be in the form of salts (see page 3, paragraph 55, lines 1 and 2).

Chiamulera et al. teaches the significant contribution of mGlu5 receptors to the behavioral effects of cocaine addiction (see page 873, column 1, paragraph 1, last 4 lines). A decrease of self-administration of cocaine was observed with an administration of the mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP); see page 873, column 2, last paragraph, lines 1-4).

To one of ordinary skill in the art at the time of the invention would have found it obvious and motivated to combine the method of Adam et al. and a combination with an

antagonist which modulates metabotropic glutamate receptor 5 because of the following: (1) Adam et al., Corsi et al., and Chiamulera et al. teach methods that treat addictive disorders; (2) Adam et al. teaches the treatment of addictive disorders with a mGluR 2 and 3 antagonist; and (3) Corsi et al. and Chiamulera et al. teach the treatment of an addictive disorder with a mGluR 5 antagonist. One would be motivated to combine the two methods because although different compounds are used and antagonize different mGluR's, they both treat addictive disorders. "It is *prima facie* obvious to combine two compositions each of which is taught by the prior art to be useful for the same purpose, in order to form a third composition to be used for the very same purpose.... [T]he idea of combining them flows logically from their having been individually taught in the prior art." *In re Kerkhoven*, 626 F.2d 846, 850, 205 USPQ 1069, 1072 (CCPA 1980). See also *In re Crockett*, 279 F.2d 274, 126 USPQ 186 (CCPA 1960); *Ex parte Quadranti*, 25 USPQ2d 1071 (Bd. Pat. App. & Inter. 1992); and *In re Geiger*, 815 F.2d 686, 2 USPQ2d 1276 (Fed. Cir. 1987).

(2) Claims 9, 27, 28 and 32 are rejected under 35 U.S.C. 103(a) as being unpatentable over Chiamulera et al. (Nature Neuroscience, 2001, vol. 4(9), pp. 873-874) in view of Adam et al. (US 6,407,094 B1) as applied to claims 1-3, 6, 7 and 16 above and Applicant's admitted prior art (see specification page 14, paragraph 2, lines 1 and 2, and page 16, group II, line 1 and 4).

The teachings of Chiamulera et al. and Adam et al. all are as applied to claims 1-3, 6, 7 and 16 above.

Chiamulera et al. and Adam et al. do not teach the antagonist 2S-2-amino-2-(1S,2S-2carboxycyclopropane-1-yl)-3-(xanth-9-yl)propionic acid (LY341495; claims 9 and 28). Also, the administration comprising: (a) administering to a subject in need thereof, an effective amount of at least one antagonist that modulates at least one of mGluR2, 3, and 5 (specifically LY341495 or/and MPEP) during a first time period, wherein the first time period is a time period wherein the subject expects to be in an environment wherein or exposed to stimuli in the presence of which, the subject habitually uses an addictive substance; and (b) administering at least one antagonist that modulates at least one of mGluR2 and/or 3 (specifically LY341495) during a second time period, wherein the second time period is a time period wherein the subject is suffering from withdrawal and/or depression, is not taught (claim 27). Lastly, wherein the first antagonist and the second antagonist are administered to the subject sequentially or simultaneously is also not taught (claim 32)

To one of ordinary skill in the art at the time of the invention would have found it obvious and motivated to combine the method of Chiamulera et al. and the antagonist LY341495 because of the following: (1) both Chiamulera et. al. and Adam et al. teach methods to treat substance abuse; (2) Adam et al. teaches the treatment of an addictive disorders or depression with a mGluR 2 and 3 antagonist; and (3) LY341495 is a well

known mGluR 2 and 3 antagonist in the art (indicated by the specification page 14, paragraph 2, lines 1 and 2, and page 16, group II, line 1 and 4). One would be motivated to combine the two methods because although different compounds are used and antagonize different mGluR's, they both treat substance abuse. "It is *prima facie* obvious to combine two compositions each of which is taught by the prior art to be useful for the same purpose, in order to form a third composition to be used for the very same purpose.... [T]he idea of combining them flows logically from their having been individually taught in the prior art." *In re Kerkhoven*, 626 F.2d 846, 850, 205 USPQ 1069, 1072 (CCPA 1980). See also *In re Crockett*, 279 F.2d 274, 126 USPQ 186 (CCPA 1960); *Ex parte Quadranti*, 25 USPQ2d 1071 (Bd. Pat. App. & Inter. 1992); and *In re Geiger*, 815 F.2d 686, 2 USPQ2d 1276 (Fed. Cir. 1987).

To one of ordinary skill in the art at the time of the invention would have found it obvious and motivated to combine the method of Chiamulera et al. and an administration comprising: (a) administering to a subject in need thereof, an effective amount of at least one antagonist that modulates at least one of mGluR2, 3, and 5 (specifically LY341495 or/and MPEP) during a first time period, wherein the first time period is a time period wherein the subject expects to be in an environment wherein or exposed to stimuli in the presence of which, the subject habitually uses an addictive substance; and (b) administering at least one antagonist that modulates at least one of mGluR2 and/or 3 (specifically LY341495) during a second time period, wherein the second time period is a time period wherein the subject is suffering from withdrawal

and/or depression; or (c) wherein the first antagonist and the second antagonist are administered to the subject sequentially or simultaneously because without unexpected results, one skilled in the art can reasonably design the period of administration.

Response to Arguments

> The Applicant argues that there is neither experimental data nor plausible substantiation in Adams et al. to suggest that the compounds are indeed effective to treating drug dependence. More importantly, as explained below, the pure speculation of Adams et al. is contradictory to results from actual scientific studies that were published in peer reviewed journals. At the time of invention, several research groups have shown that agonists (not antagonists) of mGluR2/3 were able to attenuate withdrawal symptoms and to treat morphine or nicotine dependence. Further, Fundytus et al. results show a preventative effect of the antagonist, and not efficacy in treating withdrawal if the antagonist is administered after the development of dependence. Thus, the reference teaches away from combining mGluR2/3 antagonist and a mGluR5 antagonist as presently claimed.

(1) Wrong. the presumption only applies to claimed invention in a U.S. patent - Adams et al does not claim use of mGluR 2/3 antagonists in treating addictive disorders

The Examiner disagrees because first Adams et al. is a US Patent, which is believed to be enabled by its disclosure. (2) In regards to the other art showing opposite results, the teaching of Kenny et al. (provided by Applicant's 8/31/09 in arguments) can help to explain the differences. Particularly, Kenny et al. teaches on page 1075, column 1, paragraph 3, that prolonged continuous nicotine exposure increase mGluII receptor function, but repeated exposure to psychostimulants decreased mGluII function. Thus, it

there is no such 'opposite results' because Adams et al did not show any results only speculation.

a intermittent

is possible that chronic nicotine and psychostimulant administration induce different alterations in glutamatergic transmission. Alternatevely, this apparent discrepancy may be explained by the fact that the long-term behavioral effects of drugs of abuse are related to the dosing administration regimen. In regards to the teaching away of Fundytus et al., the Examiner still believes that Fundytus et al. provides a means of attenuating withdrawal symptoms (i.e. reducing withdrawal symptoms) by administering the non-selective antagonist MCPG (see page 1018, column 2, discussion, lines 1-6; and page 1017, column 1, paragraph 3 in its entirety, Figure 1b). Thus, the non-selective mGluR antagonist MCCG (at receptors 1, 2, 3, and 5) was effective in treating withdrawal symptoms. The art provided in the rejections above support the Examiner's arguments.

Conclusion

Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not

mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

No claims allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to KENDRA D. CARTER whose telephone number is (571)272-9034. The examiner can normally be reached on 9:00 am - 5:00 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Sreeni Padmanabhan can be reached on (571) 272-0629. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

/Kendra D Carter/

Examiner, Art Unit 1627

/SREENI PADMANABHAN/

Supervisory Patent Examiner, Art Unit 1627

FORM PTO-1449 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTY DOCKET NO. TSRI 897.1	SERIAL NO. 10/527,525
		APPLICANT Markou, et al.	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT		FILING DATE 10/14/2005	GROUP 1617

U.S. PATENT DOCUMENTS

EXAM. INITIALS		DOCUMENT NUMBER	DATE	NAME	CLASS	SUB-CLASS	FILING DATE

FOREIGN PATENT DOCUMENTS

EXAM. INITIALS		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUB-CLASS	TRANSLATION YES NO

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages)

	1	Waterhouse, et al., Society for Neuroscience Abstracts 27, No. 2: 2358 (2001)
/K.C./	2	Ahmed, et al., "Neurobiological Evidence for Hedonic Allostasis Associated with Escalating Cocaine Use", Nature Neuroscience 5, No. 7: 625-626 (2002)
/K.C./	3	Harrison, et al., "Fluoxetine Combined with a Serotonin-1A Receptor Antagonist Reversed Reward Deficits Observed during Nicotine and Amphetamine Withdrawal in Rats", Neuropsychopharmacology 25, No. 1: 55-71 (2001)
/K.C./	4	Harrison, et al., "Nicotine Potentiation of Brain Stimulation Reward Reversed by DHβE and SCH 23390, but not by Eticlopride, LY 314582 or MPEP in Rats", Psychopharmacology 160: 56-66 (2002)
/K.C./	5	Cryan, et al., "Bupropion Enhances Brain Reward Function and Reverses the Affective and Somatic Aspects of Nicotine Withdrawal in the Rat", Psychopharmacology 168: 347-358 (2003)
/K.C./	6	Shigemoto, et al., "Differential Presynaptic Localization of Metabotropic Glutamate Receptor Subtypes in the Rat Hippocampus", The Journal of Neuroscience 17, No. 19: 7503-7522 (1997)

EXAMINER	/Kendra Carter/	DATE CONSIDERED	12/08/2009

EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to Applicant.